Proteomics of *Pseudomonas aeruginosa* cystic fibrosis isolates to understand host adaptation

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Dedicated to those cystic fibrosis patients who are in

a search of hope to live longer

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Declaration

I hereby certify that the work presented in this thesis titled "Proteomics of *Pseudomonas aeruginosa* cystic fibrosis isolates to understand host adaptation" is the result of my own work except where acknowledged and is not being submitted for a higher degree to any other University or Institution. Biosafety (approval number: 5201100898) approval has been duly obtained for using bacterial samples. I consent to a copy of this thesis being available in the University library for consultation, loan and photocopying forthwith.

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Abstract

Pseudomonas aeruginosa is an opportunistic bacterial pathogen commonly associated with cystic fibrosis (CF) lung infection. Numerous studies have investigated the mechanisms of adaptation of *P. aeruginosa* to CF lungs, predominantly using the laboratory reference strain PAO1 as a model organism. However, considering the vast diversity of *P. aeruginosa* strains isolated from CF patients, additional information is needed to understand the role of specific proteins in this pathophysiology. This thesis describes proteomic-oriented investigations of novel CF isolated *P. aeruginosa* strains (PASS1-4) to better understand how this organism adapts to the harsh microenvironment, typical of the CF lung.

Following genome sequencing and phenotypic profiling of the PASS strains, whole cellular proteome profiles were obtained by high-resolution mass spectrometry. Quantitative comparison of proteome profiles of PASS strains with PAO1 revealed, PASS strains share a core proteomic signature which is different to PAO1. Additionally, proteome level modulations including alteration in proteins involved in phenazine biosynthesis, aerobic respiration, PQS biosynthesis and virulence (Type 6 secretion system, hydrogen cyanide), which were reflective of genomic and phenotypic characteristics of CF isolates.

As the membrane of *P. aeruginosa* is centrally important for adhesion and virulence, iTRAQ-MS based protein quantitation of enriched cell membranes was obtained from PASS1-3 strains and PAO1, in both lung nutrient mimicking growth conditions and with a standard laboratory medium (M9-glucose minimal medium). This analysis showed decreased abundance of motility, adhesion and chemotaxis (e.g. FliK, FlgE, PilJ, PctA, PctB) proteins and elevated abundance of drug resistance (e.g. MexY, MexB, MexC) proteins. Functional assays including antibiotic MIC assay, motility and CF sputum adhesion assays confirmed the proteomic findings.

To examine how *P. aeruginosa* adapts to the hypoxic milieu, typical of CF lungs, quantitative global proteome profiles of PASS strains grown in lung nutrient mimicking medium, under 1% oxygen was performed using TMT-labelling and

SWATH-MS. Comparison of cellular proteomes with respect to normoxic baseline conditions revealed concurrent up-regulation of *cbb3* oxidases (*cbb$_3$-1* and *cbb$_3$-2*) and denitrification enzymes (e.g. NarG-I, NirC, NorB) in all strains, while CF isolates compared to PAO1 showed higher magnitude or almost exclusive expression majority of proteins, indicating PASS strains display a more robust mechanism to tackle the hypoxic stress.

In summary, this thesis describes the most comprehensive proteome profiles of clinically relevant *P. aeruginosa* strains grown in pathophysiologically relevant conditions to date. It reveals the myriad of biochemical strategies used by *P. aeruginosa* to flourish in the challenging growth environment of the CF lung.

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"To teach is to touch someone's life forever."

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Publications and conference proceedings

Peer-reviewed publications

1) *Pseudomonas aeruginosa* cell membrane protein expression from phenotypically diverse cystic fibrosis isolates demonstrate host-specific adaptations. <u>Karthik Shantharam Kamath,</u> Dana Pascovici, Anahit Penesyan, Apurv Goel, Vignesh Venkatakrishnan, Ian T Paulsen, Nicolle H Packer and Mark P Molloy. *J Proteome Res*. 2016 Jun 9.(DOI: 10.1021/acs.jproteome.6b00058)S

2) Genetically and phenotypically distinct *Pseudomonas aeruginosa* cystic fibrosis isolates share a common proteomic signature. Anahit Penesyan, Sheemal S Kumar, <u>Karthik Kamath</u>, Abdulrahman M Shathili,Vignesh Venkatakrishnan, Christoph Krisp, Nicolle H. Packer, Mark P. Molloy, Ian T. Paulsen. *PLoS One*. 2015 Oct 2;10(10):e0138527.

3) Proteomics of hosts and pathogens in cystic fibrosis. <u>Karthik Shantharam Kamath</u>, Sheemal Shanista Kumar, Jashanpreet Kaur, Vignesh Venkatakrishnan, Ian T Paulsen, Helena Nevalainen and Mark P Molloy. *Proteomics Clin Appl*. 2015 Feb;9(1-2):134-46.

Conference presentations

Poster presentation:

1) Comprehensive membrane proteome analysis of novel cystic fibrosis isolates of *Pseudomonas aeruginosa*. <u>Karthik S Kamath</u>, Dana Pascovici, Apurv Goel, Anahit Penesyan, Vignesh Venkatakrishnan, Ian T Paulsen, Nicolle Packer and Mark P Molloy. 2nd "proteomics & beyond" symposium, Macquarie university, Sydney, Australia. November 2014. (Won best poster award)

2) Comprehensive membrane proteome analysis of novel cystic fibrosis isolates of *Pseudomonas aeruginosa*. <u>Karthik S Kamath</u>, Dana Pascovici, Apurv Goel, Anahit Penesyan, Vignesh Venkatakrishnan, Ian T Paulsen, Nicolle Packer

and Mark P Molloy. MQ BioFocus conference, Macquarie university, Sydney, Australia. December 2014.

3) Membrane proteome characterization of phenotypically diverse *Pseudomonas aeruginosa* cystic fibrosis isolates reveals adaptation to host lungs. <u>Karthik Shantharam Kamath</u>, Dana Pascovici, Apurv Goel, Anahit Penesyan, Vignesh Venkatakrishnan, Ian T Paulsen, Nicolle H Packer and Mark P Molloy. 63rd ASMS conference on Mass Spectrometry and Allied Topics. St.Louis, USA. June 2015.

4) Decoding bacterial adaptation code: How does *Pseudomonas aeruginosa* adapt to the microenvironment of the cystic fibrosis lung? <u>Karthik Shantharam Kamath</u>, Anahit Penesyan, Christoph Krisp, Dana Pascovici, Apurv Goel, Vignesh Venkatakrishnan, Nicolle H Packer, Ian T Paulsen and Mark P Molloy. Human Proteome Organization (HUPO) world congress. Vancouver, Canada. September 2015.

5) Understanding adaptation dynamics of *Pseudomonas aeruginosa* in cystic fibrosis lungs. <u>Karthik Shantharam Kamath</u>, Anahit Penesyan, Christoph Krisp, Sheemal S. Kumar, Dana Pascovici, Apurv Goel, Vignesh Venkatakrishnan, Ian T Paulsen, Nicolle H Packer and Mark P Molloy. The 21st Annual Lorne Proteomics Symposium. Australia. February 2016.

Oral Presentation

Small talk: How proteins help microbes communicate. <u>Karthik S Kamath</u>, Dana Pascovici, Apurv Goel, Anahit Penesyan, Vignesh Venkatakrishnan, Ian T Paulsen, Nicolle Packer and Mark P Molloy. Three minute thesis competition. Macquarie University, Sydney, Australia. September 2014.

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Glossary of Abbreviation

AHL	*N*-acylhomoserine lactones
AES-1R	Australian epidemic strain 1R
ASL	airway surface liquid
BCA	bicinchoninic acid assay
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CID	collision induced dissociation
COG	clusters of orthologous groups
DDA	data dependent acquisition
DIA	data independent acquisition
DTT	dithiothreitol
ENaC	membrane epithelial Na^+ channel
ESI	electrospray ionization
GO	gene ontology
HILIC	hydrophilic interaction liquid chromatography
IAA	iodoacetamide
iTRAQ	isobaric tag for relative and absolute quantitation
LB	Luria-Bertani growth medium
LC	liquid chromatography
Lys-C	endoproteinase Lys-C
m/z	mass-to-charge
M9	M9 minimal medium
MS	mass spectrometry
MS/MS	tandem mass spectrometry
OM	outer membrane
OMP	outer membrane proteins
ORF	open reading frame
PAO1	*Pseudomonas aeruginosa* PAO1
PBS	phosphate buffered saline

PCA	principle component analysis
PseudoCAP	*Pseudomonas aeruginosa* community annotation project
QS	quorum sensing
Q-TOF-MS	quadrupole time of flight mass spectrometer
ROS	reactive oxygen species
RP	reversed phase
RPM	revolutions per minute
SCFM	synthetic cystic fibrosis medium
SCX	strong cation exchange
SAX	strong anion exchange
SDS	sodium dodecyl sulfate
SWATH-MS	sequential windowed acquisition of all theoretical fragmention mass spectra
TBDT	TonB dependent transporter
TMT	tandem mass tag
TOF	time of flight
UHPLC	ultra high performance liquid chromatography
WEGO	Web Gene Ontology Annotation Plot

Chapter-1

Introduction

1.1. *Pseudomonas aeruginosa*: A versatile bacterium

Pseudomonas aeruginosa is a Gram-negative, rod-shaped, facultatively anaerobic bacteria that uses a single polar flagellum for motility. It is found ubiquitously in a wide array of niche ranging from the free-living form in the environment to the human host. The *Pseudomonas* genus contains 202 known species which have been categorised based on 16S rRNA, cellular fatty acid analysis, and physiological and biochemical analyses (1). A unique feature which is predominantly found in the majority of *P. aeruginosa* strains is the production of water-soluble pigments known as pyocyanin (blue-green) and pyoverdine (yellow-green, fluorescent). Although an aerobic atmosphere is necessary for optimal growth, most strains will sustain and multiply slowly in an anaerobic environment if nitrate is present to aid respiration (2). Few, if any bacteria can match *P. aeruginosa* for its versatile metabolic adaptation capability which facilitates its survival in a vast range of challenging environments (3) (4). The bacterium has a large genome with ~5570 predicted open reading frames (ORFs) which largely aids its metabolic adaption, survival under stress and sustained virulence (5).

1.1.1. *Pseudomonas aeruginosa* is an opportunistic pathogen.

P. aeruginosa is a classic opportunistic bacterium and is able to cause infections in an array of organisms. The organisms versatile pathogenic characteristics are reflected in the wide range of organisms it is known to infect, which crosses kingdoms from animals to plants (6). *P. aeruginosa* is known to cause infections in fish, reptiles, birds, dogs, sheep and dairy herds (6). In humans, it is an etiological agent in the wide range of infections and considered one among the 'ESKAPE pathogens', thus, one of the top six infectious agents that has gained increasing attention due to alarming numbers of nosocomial infections reported worldwide (7). Being a consummate opportunistic pathogen, *P. aeruginosa* seldom causes disease in healthy individuals, yet is known to be an etiological agent in the infections of burn wounds, urinary tract infections, eye infections, cancer and noticeably in a congenital disease, cystic fibrosis (CF) (8, 9).

P. aeruginosa is notoriously renowned as a major infectious agent associated with mortality in CF airway infections. *P. aeruginosa* affected CF individuals have 2.6 times higher risk of death compared to non-infected counterparts (10). Hitherto, reasons for the specific acquisition of *P. aeruginosa* in CF patients are a matter of conjecture. Initial colonising strains are known to be acquired from the environment (11) and may be displaced by more virulent and transmissible epidemic strains through patient-to-patient contact (12, 13).

1.2. **Cystic fibrosis**

Cystic fibrosis (CF) is a congenital, autosomal recessive, debilitating disease, most frequent in Caucasian population affecting ~70,000 individuals worldwide (14). CF is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR gene mutation was discovered by positional cloning which identified the gene deletion on the chromosome seven (15). A fully functional CFTR regulates the chloride channel in a cyclic adenosine monophosphate (cAMP) dependent way. Key understanding of the pathophysiology of CF was derived by understanding several mutants of CFTR gene (16). To date, over 2000 CFTR gene mutations have been reported in the cystic fibrosis mutation database (17), however, F508del, which refers to deletion of the phenylalanine codon at position 508, accounts for approximately 70% defective CFTR alleles (18). All subjects diagnosed with CF have mutations in both CFTR gene alleles. Hence, gene mutation test along with sweat chloride test is routinely used for diagnosis of the disease (19)

1.2.1. Sequelae stemming from the dysfunctional CFTR

Aberrant CFTR protein is a product of mutations in the CFTR gene which may lead into one of six phenotypes (20) namely; 1) absence of synthesis of CFTR protein 2) defective protein maturation and premature degradation 3) aberrant regulation, such as less ATP binding and hydrolysis. 4) defective chloride conductance 5) low number of CFTR transcripts due to a promoter or splicing abnormality and 6) an accelerated turnover from the cell surface, each of which may ultimately lead to a dysfunctional or absence of CFTR protein.

CFTR protein is expressed in many epithelia across the body including airway surfaces, pancreas, sweat glands, gut, neurons and other fluid-transporting tissues (21) and pivotal in the anion transport mainly chloride, sodium and bicarbonate ions across the epithelial membrane. Secretion of chloride by CFTR establishes an electrical driving force for transmembrane secretion of sodium through epithelial sodium channels (ENaC). This movement collectively generates an osmotic driving force for water to flow to the exterior of the cell (22). Aberrant CFTR expression in the apical membrane causes an imbalance in fluid homeostasis ultimately resulting in an elevated absorbance of water by epithelial cells, leaving the exterior surface dehydrated (Figure-1.1A-B). This event manifests various abnormalities across multiple organs in the body which heavily rely on an exocrine function including lungs, pancreas, intestine, liver, vas deferens and sweat glands (Figure-1.1C).

In the lungs, the CFTR mutation leads to depletion of water from the airway surface liquid (ASL) layer of the conducting airways resulting in thick and viscous mucus, ultimately leading to decreased respiratory function (discussed in detail in section: Effects of CFTR mutation on lungs). One of the fundamental defects observed in the CF patients, independent of the central theory of abnormal secretion of mucus, is elevated levels of chloride ions in the sweat, so high that the sweat chloride level detection is the most widely used and reliable test for the diagnosis of CF (23). This is due to defects in reabsorption of salt and water manifested as a result of dysfunctional CFTR protein.
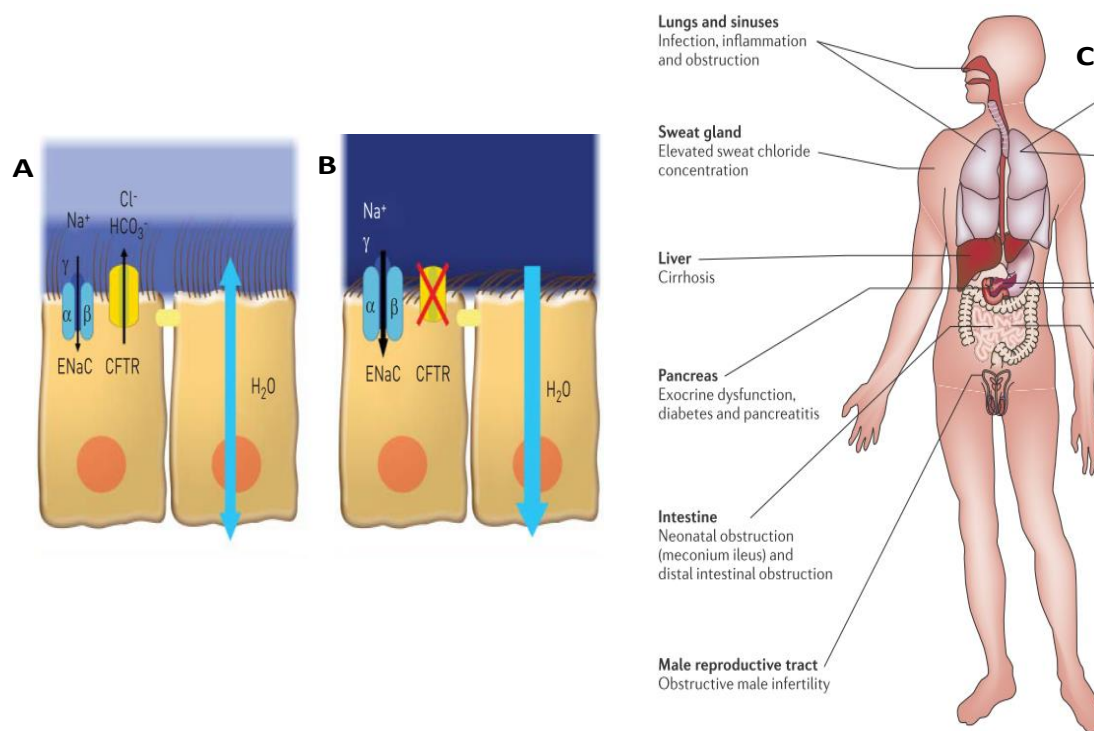
Figure-1.1: Dysfunctional CFTR, aberrant water homeostasis, and affected organs.

(A) A fully functional CFTR protein permits transmembrane transport of chloride and bicarbonate to exterior of the cell, leading to the transport of sodium ions into the cells, ultimately generating osmotic driving force leading to transport of water to exterior of the cells causing hydration of the external surface of the cells (light blue coloured surface) (B) Dysfunction/absence of CFTR protein fails to transport chloride and bicarbonate sequentially leading to excessive water absorption and dehydrated external surface (dark blue coloured surface).(C) Dysfunctional CFTR proteins cause malfunctioning of several organs in the body (14, 24).

1.2.2. Effects of CFTR mutation on the lungs

Pulmonary disease in CF is a challenging complication in the management of the disease and determinant of quality of life. Obstructive lung disease is currently the major cause of morbidity and is responsible for ~80% of mortality (14). Unlike other organs affected by CF, lungs portray an intricate pathophysiology which is a combination of CFTR mutation, inflammation (endogenous factors) and infection by pathogens (exogenous factor), all of which collectively lead to the irreversible damage to the lungs.

Healthy human airway epithelium is covered with a thin layer of liquid called airway surface liquid (ASL). The ASL can be subdivided into two layers according to

viscosity; lower, periciliary liquid (PCL) and upper mucous blanket. Less viscous, watery PCL is about 7μm thick and consists of water, salt and covers the cilia originating from the ciliary epithelium. The upper highly viscous layer, apart from water (~98%) and salt, mainly consists of highly glycosylated, slimy gel-like protein mucin (~1%) secreted by goblet cells and by Clara cells in the terminal bronchioles (25, 26). An upper layer mucus gel traps inhaled particles and the lower PCL layer with the help of ciliary beating, propels mucus along with trapped particles towards the mouth at the rate of ~3mm/min (27), where it can be expectorated or swallowed. This process is termed as mucociliary clearance and it is one of the first lines of defence used to clear inhaled particles, preventing most infections.

Normal mucociliary clearance requires a fine balance between the composition and volume of the mucus. In the case of CF, increased reabsorption of the water leaves ASL dehydrated. Further, polymers including bacterial biofilms, DNA, proteoglycans and filamentous actin, along with infectious agents and inflammatory cells, increase the complexity of the PCL. Chronic bacterial infections cause increased production of gel-forming mucins (increased levels of MUC5AC and MUC5B by 908% and 59%, respectively), making the sputum highly viscous (28). Change in molecular composition severely affects the PCL resulting in thick and dehydrated mucus accumulated on the airway surface in the form of hypoxic mucus plugs. The mucus plugs block the airways and are often difficult to eradicate. Further, the mucus plugs create a good niche for trapped pathogens which in the case of a normal individual is cleared using mucociliary clearance mechanism (29).

In summary, the CFTR mutation in epithelial cells of lungs leads to an imbalance in water homeostatic leading to secretion of thick mucus impairing mucociliary clearance ultimately leading to infection and inflammation.

1.2.3. Beyond the mutation: Infections associated with CF lung disease.

A palpable feature of CF is the persistent airway infection which is extremely difficult to eradicate through antimicrobial therapy. Hence, these pathogens are

virtually suppressed and never completely eradicated. There are many hypotheses connecting defects in CFTR with susceptibility to microbial infection.

The salt depletion hypothesis: This hypothesis assumes that the CFTR malfunction leads to the increased salt concentration in the ASL, concomitantly leading to inactivation of defensins. The theory was supported by a work by Smith *et al* (30) which through in vitro experiments showed inactivation of defensins in salt concentrations higher than 50 mmol/L thereby facilitating infection and bacterial growth.

On the contrary, according to the isotonic low volume hypothesis, the infection occurs due to reduced water volume in ASL which entraps the infectious agents (29). This theory is also referred to as "the low volume hypothesis". It is one of the most widely accepted theories which explains most of the components of the pathophysiology of the CF.

A third proposal by Teichgräber and colleagues (31) demonstrated the relationship between ceramide and increased predisposition to bacterial infection, specifically *P. aeruginosa* infection in CF mouse models. They hypothesised that the accumulation of ceramide in an age-dependent manner leads to alkalization of intracellular vesicles in CFTR deficient epithelial cells leading to pulmonary inflammation, cell death and high susceptibility to *P. aeruginosa*. Interestingly, the accumulation was normalised in mice with genetic deficiency in sphingomyelinase gene (involved in cleavage of sphingomyelin to ceramide) ultimately reducing susceptibility to infection.

All three theories address the issues of the predisposition of CF patients to infection on a cause and effect basis, where, a defect in CFTR leads to altered cellular events such as, change in the salt and water content, which concomitantly exposes the cells to the infection, rather than being a direct cause. Notwithstanding the foregoing, these theories do not illustrate the reasons for predisposition of CF patients to a specific subtype of pathogens, for instance, *P. aeruginosa*.

1.3. Relationship between CF and *P. aeruginosa*

A number of hypotheses try to demonstrate a direct relationship between CF and *P. aeruginosa*. The first theory illustrates the role of CFTR as a receptor for the internalisation and clearance of the *P. aeruginosa*. Hence, in the case of CF, mutant CFTR leads to reduced binding and lowered clearance of the bacterium from the airway surface (32-34). On the other hand, some of the studies showed that the CF affected cells have increased adherence to pathogenic bacterium on the apical surface through dedicated receptors, leading to increased association of *P. aeruginosa* with CF-affected epithelial cells (35, 36). Further, Zar and colleagues (37) using CF patient-derived cells with various CFTR mutations demonstrated that the patients with the homozygous mutation for the delta F508 have a significantly higher binding capacity to *P. aeruginosa* in comparison to their counterparts, heterozygote carriers or healthy individuals.

Considering these theories, although it is very tempting to make a conclusion that the CFTR is the sole reason for predisposition of bacterial infection, some exceptions state otherwise. For instance, majority of the studies are conducted using the model organism *P. aeruginosa* and do not explain much about other infectious agents including common bacteria such as *Burkholderia cepacia complex* (BCC), *Staphylococcus aureus*, and fungi. Hence, it is safe to interpret that multiple factors collectively influence the association of *P. aeruginosa* with CF.

1.3.1. Microbial flora of the CF respiratory tract

Growing evidence suggests that the CF airways harbor a high load of a diverse, polymicrobial community which is medically challenging to treat (38-41). Since the infections adversely affect the quality of life of the CF patients through periodic episodes of chronic pulmonary exacerbations and inflammation, there have been several attempts to understand the dynamics of the microbial population.

The traditional view of CF microorganism infection is that the bacterial species are acquired in a temporal succession, starting early in life. Typically, this includes well-known species, such as *S. aureus*, BCC, *Haemophilus influenza* and *P. aeruginosa* (42).

However, with advancement in genetic profiling technology for species identification we see that the traditional view is being replaced, detailing a more complex polymicrobial community comprising of bacteria, fungi and viruses. A detailed list of pathogens often associated with CF is listed in Figure-1.2 and Supplementary Table-9.1.

Longitudinal studies have demonstrated that the CF lung microbiome diversity decreases with the increasing age with *P. aeruginosa* dominating in adult patients, ultimately leading to deterioration of the lung function (Figure-1.3A-B), however, a specific mode of acquisition of the bacterium, still remains unknown. Microbial infections play a vital role in causing irreversible damage to lung airways directly through microbial (eg: bacterial toxins) secretions and indirectly through initiating an exaggerated host inflammatory response. It is important to understand both of these causes to develop a therapeutic intervention for the disease.

Figure-1.2: List of microorganisms commonly identified in the respiratory tract of CF-affected individuals. Figure adopted from Magalhães *et al*(43).
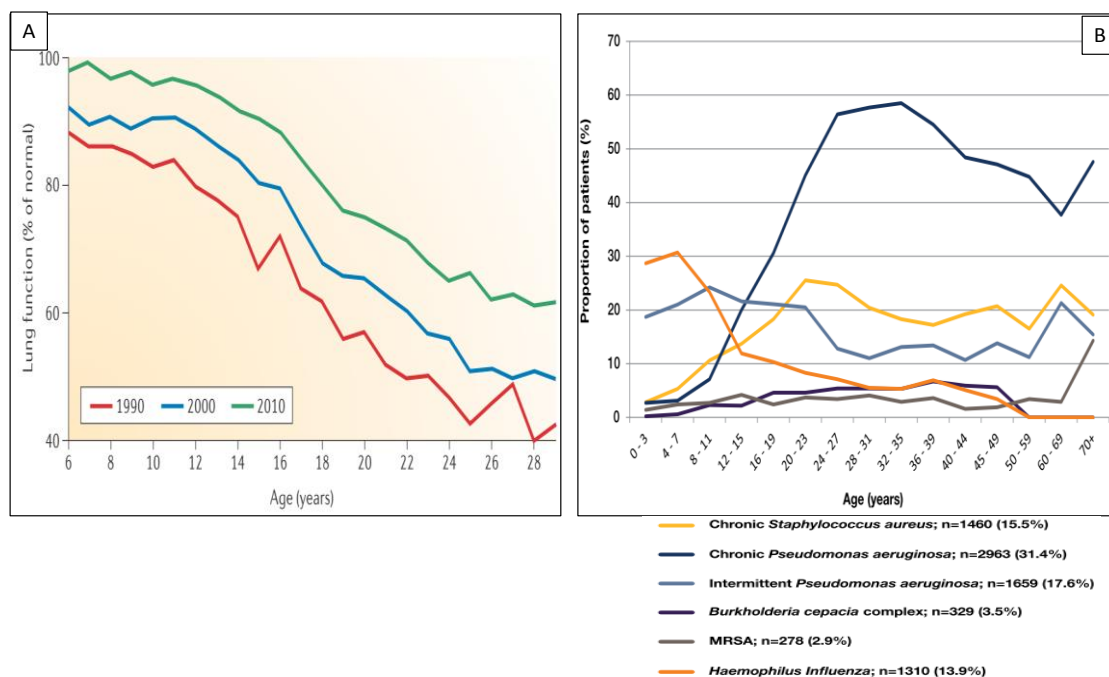
Figure-1.3: Relationship between microbial infection and diminishing lung functions in CF. (A) As age progresses, lung function diminishes. (Adopted from Folkesson *et al* (44)). (B) Infection in lungs of CF patients during inception is polymicrobial in nature, with progression, *P. aeruginosa* dominates the colony. (adopted from UK CF Registry scientific national report 2013 (45)).

1.3.2. Infection and inflammation: a chicken-and-egg conundrum

A key observable feature of CF is an exaggerated inflammatory response in the lungs. Innate immunity of the CF-affected individual acts as a "double-edged sword", where it is both constructive and destructive at the same time. Several factors including the bacterial shedding of pathogen–associated molecular patterns (PAMPs), cell wall components, lipopolysaccharides (LPS), flagella, DNA are known to stimulate the inflammatory response constructively. High expression of pro-inflammatory cytokines including tumour necrosis factor-alpha (TNF-α), interlukin-8 (IL-8), interlukin-6 (IL-6), interlukin-17 (IL-17) has been observed in CF airways (46). These pro-inflammatory cytokines induce recruitment of the macrophages and neutrophils into the airway, which in turn release more pro-inflammatory mediators and chemoattractants. Thus, a perpetuating, inflammatory response is established which is a chief contributor to the destruction of lung tissues and deterioration of lung function.

The relationship between infection and inflammation in CF could be compared to the "chicken and egg" conundrum, as it is debatable whether infection leads to inflammation or vice versa. Compelling amount of evidence suggests that an infection triggers the airway inflammation. Studies using bronchial epithelial cells in cell culture (47), showed little or no IL-8 or NF-κB activation in the absence of inflammatory stimulus. Similarly, BAL fluid profiles for pro-inflammatory cytokines were found similar when measured in CF infants and age-matched, non-CF control subjects (48). These studies provide evidence for the absence of an inflammatory response before the pathogenic stimulus and suggest that the inflammation may be triggered on encountering infectious agents.

On the contrary, the preponderance of evidence suggests an active, inherent inflammatory response, independent of infection, evidenced by the accumulation of free proteases and polymorphonuclear neutrophils (PMN) well before evidence of detectable infection (49). Weber *et al* showed activation of NF-κB followed by IL-8 transcription independent of infection and manifestation of "cell stress" as a consequence of the accumulation of defective CFTR in the endoplasmic reticulum (50). Notably, Rosenfeld and colleagues have shown the presence of elevated levels of proinflammatory cytokines and neutrophils in BAL fluids of infants and children with CF in the absence of clinically detectable respiratory infections (51). However, the inflammatory response might have been due to subclinical infections occurred before the collection of BAL. Also, viral infections, which are frequently detected in lungs of CF patients (52), might be responsible for triggering the proinflammatory response.

1.4. *P. aeruginosa* colonisation of the CF lung

Several factors contribute to the successful adaptation to the host and prevalence of *P. aeruginosa* in CF. Several key features involved in the evolution of *P. aeruginosa* as a successful pathogen are summarised in Table-1.1 and explained in the following sections, 1.4.1-1.4.6.

Table 1. 1: Examples of adaptation strategies utilised by *P. aeruginosa* to the host during chronic infection and general outcome. (Table adopted from reference (53))

Bacterial component	Adaptation	Effect of adaptation	Reference
Genome	Mutations (single nucleotide polymorphism, insertion, deletion)	Renders selective advantage through metabolic adaptations, drug resistance among others	(54)
Lipopolysaccharide	Loss of O-antigen, production of lipid-A variants	Contributes towards host immune system evasion	(55)
Exopolysaccharide	Non-mucoid to mucoid switch	Host immune system evasion, protection against oxidative burst, resistance to phagocytosis	(56)
Motility	Loss of flagella, pili and chemotaxis	Evasion of phagocytosis.	(57, 58)
Colony morphotypes	Conversion to small colony variants	Resistance to phagocytosis, stronger adherence to surface, increased production of exopolysaccharide	(59)
Quorum sensing	*lasR* and *rhlR* mutation	Helps to avoid cell lysis.	(60)

1.4.1. Genome complexity of *P. aeruginosa* acts as an instrument for host adaptation

Compared to most other pathogenic bacteria, *P. aeruginosa* has a relatively large and complex genome ranging from 6.22 to 6.91 Mb in size (54). To date, 26 *P. aeruginosa* fully sequenced genomes are publically available in *Pseudomonas* Genome DB (*http://www.pseudomonas.com*) (as of 27th Jan 2016), of which, 16 are isolated from the human host and specifically, five are from CF individuals (61).

Genome analysis provides valuable insights into survival and pathogenesis strategies *P. aeruginosa.* The *P. aeruginosa* pan-genome consists of two parts, a core genome accounting for about 90% of genomic content which is commonly conserved between strains and an 'accessory' genome, uniquely found in few strains. The core genome

supports the adaptation through rearrangements, deletion, and mutation. On the other hand, the accessory genome largely encompasses genes received through horizontal gene transfer and acts as a primary contributor in micro-evolution of the genome. For instance, sustaining in the host through encoding novel metabolic pathways (62), encoding virulence factors (63) and encoding genes for resistance to multiple classes of antibiotics (64) are mainly aided by accessory gene pool. Notably, the highly plastic *P. aeruginosa* genome encodes for a disproportionately large number of outer membrane proteins involved in sensing the environment, nutrient uptake, antibiotic efflux, virulence factor transport, adhesion and motility, which, not surprisingly allows the bacterium to thrive under harsh and diverse conditions (62). Additionally, the *P. aeruginosa* PAO1 genome encodes about 127 two-component systems (TCS) members, quintessential for sensing the external environment for activation of specific transcriptional regulators. It is marginally very high compared to 60 TCS in *E. coli* and 70 in *Bacillus subtilis*, reflecting the dynamic adaptability of *P. aeruginosa* (65).

Of note, the *P. aeruginosa* genome has the third largest regulatory network ever reported in bacteria with ~690 regulatory genes and 1020 regulatory interactions (12% of total genes), which orchestrates expression of a battery of genes (66). Additionally, *P. aeruginosa* strains exhibit up to 1000-fold increase in the spontaneous rate of mutation during infection (67), aided by down-regulation of mismatch repair system enzymes MutA, MutL, and UvrD, helping the bacterium respond to selective pressures (67, 68). For instance, Smith *et al* (69) identified 68 mutations in a *P. aeruginosa* strain during eight years of infection. One of the key mutations which is associated with the poor prognosis of CF patients was an over-production of exopolysaccharide (alginate) and conversion to the mucoid phenotype. Put together, the plasticity encoded within the large *Pseudomonas* genome enables the bacterium to respond and adapt to new and challenging environments.

1.4.2. Quorum sensing and virulence

Quorum sensing (QS) is a cell density dependent intercellular signalling system, facilitated by small diffusible molecules called autoinducers (AIs), which control the expression of more than 20% of the expressed bacterial proteome (70). There are three major quorum sensing systems in *P. aeruginosa*. Two of the well-characterised system *las* and *rhl* rely on N-acyl homoserine lactone (AHL) and the third one is mediated by the alkyl quinolones, hence named *Pseudomonas* quinolone signal (PQS). All three systems are intertwined and act in a hierarchical way (71) and are known to control a vital set of genes involved in the pathogenesis including virulence (72), pigmentation, biofilm formation, drug efflux, immune evasion and nutrient intake (73).

One of the crucial functions, biofilm formation, has been strongly linked to QS system. Davies and colleagues through *in vitro* experiments (74) showed that *lasI* mutants lacked the capacity of forming a structurally stable biofilm indicating the importance of QS in the formation of biofilms. Interestingly, similar trends of reduced virulence, tissue destruction and decreased mortality of *rhlI* and *lasI* mutants of *P. aeruginosa* compared to wild-type strains, were observed in multiple animal infection models (75). CF patients' sputum with *P. aeruginosa* infection was found to have significant concentrations of AI, N-3-oxododecanoyl homoserine lactone ($3OC_{12}$-HSL) and N-butyryl-L-Homoserine lactone (C_4-HSL). This may indicate the active participation of *P. aeruginosa* QS system in colonisation of the CF airways.

The virulence factors are an indispensable part of the pathogenesis of *P. aeruginosa*. The bacterium expresses a broad arsenal of virulence determinants involved in the colonisation, immune response inhibition and cytotoxicity under the QS regulation (76). For instance ToxA, a virulence factor, secreted by type two secretion pathway, among others, was shown to be controlled by the *lasR* system in *P. aeruginosa* strains isolated from the CF patients' sputum (77). Similarly, anti-host effector toxin ExoS, which is translocated into the eukaryotic cells by a type III secretion pathway, was shown to be regulated by *rhlR/rhlI* QS system (78).

Table 1. 2: Examples of *P. aeruginosa* virulence factors controlled by QS and their effect on the host. (Table adopted from reference (76))

QS-regulated gene	Protein/ virulence factor	Effects to the host during infection	Benefits to *P. aeruginosa*	References
lasB	Elastase	Degradation of elastin, collagen, and other matrix proteins	Extracellular iron acquisition from host proteins	(79, 80)
lasA	Protease	Disruption of epithelial barrier	Staphylolytic activity, host immune evasion and enhanced colonisation	(81)
toxA	Exotoxin A	Cell death	Establishment of infection; enhanced colonization	(82)
aprA	Alkaline protease	Degradation of the host complement system and cytokines	Immune evasion and Persistent colonization	(83)
rhlAB	Rhamnosyl-transferase (Rhamnolipid)	Necrosis of the host macrophages and polymorphonuclear lymphocytes	Immune evasion; biofilm development	(84, 85)
lecA	Lectin	Paralysis of airway cilia	Establishment of infection; enhanced colonization	(86)
hcnABC	Hydrogen cyanide	Cellular respiration arrest; Poorer lung function	Enhanced colonization	(87)
phzABCDEFG, phzM	Pyocyanin	Oxidative effects dampen host cellular respiration and cause oxidative stress; paralysis of airway cilia; delayed inflammatory response to *P. aeruginosa* infections through neutrophil damage	Establishment of infection; enhanced colonisation; immune evasion	(88-90)

Furthermore, *in vitro* assay by Gambello *et al* showed that a *lasR* isogenic mutant PAO strain, displayed reduced levels of exotoxin A, implicating the key role of QS in the expression of virulence factors (91). List of major virulence factors expressed by *P. aeruginosa* under the control of QS and their benefits to the bacterium has been summarised in the Table-1.2.

Apart from controlling bacterial cellular traits, AIs have also been shown to interact with the host cells directly and change cell physiology and function in an immunomodulatory way. *In vitro* experiments (92, 93) have shown that the treatment of human bronchial epithelial cells with purified $3OC_{12}$-HSL, induced secretion of inflammatory cytokine IL-8. Further, experiments demonstrated activation of multifarious inflammatory mediators in the mouse model upon injection of purified $3OC_{12}$HSL into the skin (94). Contrastingly, AIs have also been demonstrated to act as immunosuppressors. $3OC_{12}$HSL have been shown to down-regulate the host expression of IL-12, tumour necrosis factor alpha (TNF-α), inhibit lymphocyte proliferation, activate T cells to produce gamma-interferon and induce accelerated apoptosis of macrophages and neutrophils (94-96). Additionally, PQS, another AI, has been shown to actively suppress the host immune response expression mediated through NF-κB pathway (97). The *pqsA* gene mutants, that do not secrete the AI, failed to disseminate in the mouse lung tissues (97), suggesting a key role of PQS QS in the pathogenesis of *P. aeruginosa*.

Interestingly, QS-negative mutants, that produce little or no AI, are often isolated from the CF patients (98). It has been hypothesised that these mutants act as "social cheaters", where QS-deficient strains benefit from co-colonising QS-proficient strains and the former avoids the synthesis of QS molecules since it is a "metabolic burden"(99). Although such mutation renders reduced virulence, till date, it is unclear, why such QS mutant strains are evolutionarily maintained. Interestingly, QS mutants have been shown to be better equipped to avoid cell lysis and death (60). This may indicate the *P. aeruginosa* virulence is multifactorial and players beyond QS may potentially help *P. aeruginosa* in pathogenesis (100).

In conclusion, QS plays a vital role not only in governing complex traits important in the pathogenesis of *P. aeruginosa* but also by directly affecting the host my manipulating cellular functions. Thus QS provides a survival advantage to *P. aeruginosa*, which undoubtedly, makes it an ideal target for the antagonist.

1.4.3. Evasion of the host immune system:

Once within the host, *P. aeruginosa* is constantly under struggle with the host immune system. The host immune system recognises the presence of *P. aeruginosa* through a family of pattern recognition receptors (PRRs) and activates the immune system to clear it through detection of pathogen-associated molecular patterns (PAMPs) expressed by the bacterium (101). However, the bacterium plays "molecular stealth" and alters the PAMPs to subvert the immune system recognition. One of the widely reported responses is down-regulation of immunostimulator, flagellin (FliC) synthesis which forms the building blocks of flagella. Such an event helps *Pseudomonas* to evade TLR-5 mediated phagocytosis (102, 103).

One more distinct characteristic of *P. aeruginosa* adapted to the CF lungs is a modification in the membrane lipopolysaccharides (LPS). Lipid A, an integral component of LPS, mediated immunostimulatory action mainly through CD14, TLR4, and the secreted protein component MD-2 (104). In-depth mass spectrometry and NMR characterisation of Lipid A of CF isolates of *P. aeruginosa* revealed, that they were uniquely modified and such modifications were absent in the laboratory-adapted strains, non-CF clinical isolates from the acute clinical infections (blood, ear, eye, urinary tract) and isolates from bronchiectasis (105). Specifically, lipidA of *P. aeruginosa* CF strains were modified with the addition of aminoarabinose and palmitate, which conferred resistance to host immune defences including cationic antimicrobial peptides (105).

Taken together, modifications of *P. aeruginosa* PAMPs plays an essential role in evading the host immune recognition and establishing a favourable condition for the long term persistence of the chronic infection.

1.4.4. Drug resistance:

One of the main contributors for persistent *P. aeruginosa* in CF is its resilience towards a wide range of antibiotics, which makes the management of the disease much challenging. "Pan-resistance" of *P. aeruginosa* to a wide array of antibiotics could be due to intrinsic or acquired mechanisms (Table-1.3). The intrinsic mechanisms predominantly include 1) low permeability of the cell membrane 2) naturally occurring genes on the bacterial chromosomes, such as ß -lactamase 3) biofilm formation 4) efflux pumps. Acquired factors include, 1) acquisition of resistance genes via transposons, bacteriophages, plasmids and other mobile elements (106) 2) mutations in the genes targeted by antibiotics. 3) changes in membrane physiology.

P. aeruginosa intrinsic resistance mechanism is exemplified by relatively high impermeability of the outer membrane. It is ~92% less permeable with a molecular size exclusion limit of ~3000 Da which is higher when compared to the Gram-negative bacterium *E. coli* with an exclusion limit of ~500 Da (107, 108). This size exclusion effectively retards majority of the antibacterial agents presently used for the treatment of the bacterium. Furthermore, if the antibiotic containing ß -lactam ring such as penicillin, penetrates the outer membrane, an innate, periplasmically located enzyme ß-lactamase inactivates the antibiotic, thus playing a central role in the intrinsic resistance of *P. aeruginosa*. Another significant contributor to the intrinsic resistance of *P. aeruginosa* is widely distributed efflux pumps which could be specific to a substrate, or could export structurally diverse and unrelated molecules. For instance, MexAB-OprM root–nodulation–cell division (RND)-type efflux pumps have been linked to resistance to a wide class of structurally unrelated compounds including aminoglycoside (gentamicin) and home cleaning and hygiene product bisphenol compounds (109, 110). Up-regulation in the expression of efflux pumps including multi-drug efflux pumps MexAB-OprM and MexCD-OprJ was commonly seen associated with resistance to drugs including aminoglycosides, quinolones, and carbapenems (111).

Acquisition of the resistance commonly occurs through conjugation (conjugative transposons and plasmids), transduction assisted by bacteriophages and transformation which includes incorporation of DNA (particularly for drug resistance) from a dying organism into the recipient bacterial chromosome or plasmid DNA (106). The classic example of mutation based drug resistance in *P. aeruginosa* is the resistance to fluoroquinolone which acts on the DNA gyrase (encoded by *gyrA*) and topoisomerase IV, thereby attacking the cellular DNA replication machinery. *P. aeruginosa* protects itself from the fluoroquinolone by mutating *gyrA gene.* In vitro mutational analysis showed 128 times higher fluoroquinolone resistance in *P. aeruginosa* with mutation *gyrA(112)*. Additionally, high frequency of *gyrA* mutation was observed in the clinical strains of *P. aeruginosa* resistant to fluoroquinolone (113).

Understanding of the antibiotic targets and resistance mechanism has significantly contributed to the increased survival rate of the CF patients by controlling bacterial infections in CF lungs. U.S. cystic fibrosis foundation patient registry annual data report suggests a median increase of life expectancy of CF patients to be 37 years compared to 25 years in 1985 (114). However, strains unresponsive to all antimicrobial drugs are now coming to light, which underscores a need for better management of antibiotic administration and the need for discovery of new classes of drugs with better efficacy.

Table 1.3: Commonly known drug resistance mechanisms in *P. aeruginosa* (Table adopted from reference (8))

Resistance mechanism	Mechanism of drug resistance	Proteins involved	Example drugs
Intrinsic	Efflux pumps	MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM	Cephalosporins, carbapenems, aminoglycosides, quinolones, ureidopenicillins
Intrinsic	Outer membrane impermeability	Opr-F, Opr-D, Opr-B	Carbapenems, aminoglycosides, quinolones
Intrinsic	Enzymatic digestion of antibiotic	AmpC (β-lactamase)	Penicillins
Acquired	Targeted mutation	DNA gyrase, DNA topoisomerase, MexZ	Quinolones, cefapimes, aminoglycosides
Acquired	Horizontal gene transfer	Metallo-β-lactamse, Extended-spectrum β-lactamases (ESBLs)	Penicillins, cephalosporins and carbapenems
Acquired	Membrane changes	Lipid-A modifications	Aminoglycosides and polymyxins

1.4.5. Adaption to extreme stresses: Hypoxia and oxidative stress

"….. The history of evolution is that life escapes all barriers. Life breaks free. Life expands to new territories, painfully, perhaps even dangerously. But life finds a way". -Dr. Malcom in Jurassic Park (Michael Crichton) (115).

During the establishment of infection in the lungs of CF patients, *P. aeruginosa* adapts to the multiple external stresses including limited availability of oxygen (hypoxia) and the presence of oxidative agents, osmotic stress among others. Steep oxygen gradients are present in the mucus on CF epithelial surfaces prior to any detectable infections (116, 117). Worlitzsch and colleagues through *in situ* studies on lungs of CF patients undergoing transplant concluded that the successful invasion of *P.*

aeruginosa would require it to move from an aerobic condition to microaerobic or anaerobic conditions inside mucus plugs (116). In such conditions, *P. aeruginosa* switches to respiration using inorganic terminal electron acceptor, nitrate (NO_3) and nitrite (NO_2), nitric oxide (NO) or nitrous oxide (N_2O), replacing oxygen in the electron transport chain. In non-availability of nitrate or nitrite, arginine is degraded to ornithine, although it poorly serves as an energy substrate for generation of ATP (118). Although it cannot be a standalone sustainable source of energy, pyruvate fermentation is used in the anaerobic growth, which provides a metabolic capacity to the bacterium for long-term survival of up to 18 days (119).

The *nar, nir, nor* and *nos* gene clusters encode a set of reductases required for reduction of nitrate to nitrogen gas through intermediates nitrite, nitric oxide and nitrous oxide (120). Nitrate concentration has been measured in the CF sputum to be ranging from 10 to 700 μM, adequate to sustain the limited growth of the bacterium (121) (118).

Adaptation to the anaerobic conditions brings about significant changes in *P. aeruginosa* physiology which results in the increased production of virulence factors including cyanide, alginate, and exotoxin A and elevated resistance to the antibiotics including in the biofilm mode (124-127). Nevertheless, still there is a constant debate about whether *P. aeruginosa* growth in CF lungs is aerobic or anaerobic (128). A contrasting view to existing theory of distinct metabolic regulation of *P. aeruginosa* under anaerobic conditions was provided by Alvarez-Ortega *et al*. Through transcriptome and phenotype studies of *P. aeruginosa* strain PAO1 grown in completely aerobic, microaerobic and anaerobic conditions they demonstrated that, there is an overlapping set of genes that control sustenance of the bacterium under all three conditions. These results indicate that *P. aeruginosa* responds to alterations in oxygen concentration along a continuum rather than having a distinct low oxygen regulation system. Considering heterogeneity of the oxygen distribution in the lung micro-environment, reaching to a concrete conclusion still remains elusive.

In the hostile CF lung milieu, *P. aeruginosa* is continuously exposed to both endogenous and exogenous fluxes of cytotoxic oxidants. One of the leading causes of the oxidative environment is an overwhelmed inflammatory response in the CF lungs, exemplified by soaring high levels of neutrophils, ~1500 times more than healthy individuals (129). Activated neutrophils and degranulation generate a heavy load of reactive oxygen species (ROS) along with myeloperoxidase, neutrophil elastases and reactive nitrogen species (RNS), all of which collectively impose oxidative stress to *P. aeruginosa*. Additionally, pyocyanin, an endogenous redox active molecule, depletes cellular antioxidants thus increasing the oxidative damage to the bacterium (130).

ROS in the lungs includes superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO•) which exert mutagenic stress on the bacterial DNA. *P. aeruginosa* responds to the stress using several endogenous free radical scavenging enzymes and production of exopolysaccharide, alginate (131). Free radical scavenging enzymes produced by *P. aeruginosa* include superoxide dismutases (SodB and SodM with iron and manganese in active site respectively) and catalases (encoded by *katABCF*) (132). On the contrary, oxidative stress imposes a selective pressure which makes the bacterium generate more alginate, grow insensitive to antibiotics and ultimately lead to the high frequency of generation of hypermutable isolates (133). Hence, an obvious strategy for prevention of hypermutable isolates is to potentially administer antioxidants. Chopra *et al* (134) showed a reduction of mutation frequency of hypermutable strains of *E. coli* on the addition of exogenous antioxidants to the culture. Additionally, administration of the antioxidant, N-acetylcysteine (NAC) to the CF patients, reduced the oxidative burden caused due to polymorphonuclear leukocytes (PMNs) and monocytes and had a positive impact on the clinical condition of patients (135). However, direct effects of NAC on *P. aeruginosa* burden in the CF patients were not reported in the study and warrants further investigation.

This indicates oxidative stress imparted by the host immune system is a "double-edged sword". When under control helps to eradicate infectious agents but when overwhelmed causes harm to the self and induces hypermutable, more evolved strains of the pathogens including *P. aeruginosa* which are hard to eradicate completely.

1.4.6. *P. aeruginosa* PASS strains as model organism to study adaptation of the bacterium to CF lungs:

P. aeruginosa strain PAO was isolated from a burn wound patient, in Bruce Holloway's laboratory in Melbourne Australia in 1955 (136). Strain PAO1 is a spontaneous chloramphenicol-resistant mutant variant of the original PAO strain (137). Several sublines of PAO1 have been passaged in laboratories across the world including, PAO1-UW, whose complete genome was sequenced in 2000 (5). It revealed relatively large sized genome comprising of 6.3 million base pairs, with 5,570 predicted open reading frames. Several genomic and phenotypic variations have been reported with in sublines of PAO1 strains (137). For instance, comparison of genome sequence of PAO1-UW with the physical map of original PAO1 strain revealed a large, 2.2-Mb inversion (5). Furthermore, PAO1-D and PAO1-J which are German and Japanese sublines of PAO1 respectively, were found to be quorum-sensing negative mutants which carried point mutations in the regulatory gene *las*R (60).

Owing to large diversity in the phenotype and genome (137), choice of PAO1 as a model organism to study CF pathophysiology, is questionable. On the other hand, *P. aeruginosa* PASS strains (PASS1, PASS2, PASS3 and PASS4), used in this thesis, were freshly isolated from sputum of the CF patients of diverse gender, age group and antibiotic treatment (Table-1.4) (138).

Table-1.4: Details of *P. aeruginosa* PASS strains used in the study (138).

Strain	Biological source	CF genotype	Patient Age, Gender	Number of predicted ORFs	Genome size (Mbp)	Similarity of genome to PAO1 (%)
PASS1	CF patient's sputum	ΔF508/ΔD1507	40, female	5792	6.3	89.12
PASS2	CF patient's sputum	ΔF508/ΔF508	27, male	5795	6.1	88.18
PASS3	CF patient's sputum	ΔF508/ΔF508	23, male	5847	6.4	88.8
PASS4	CF patient's sputum	ΔF508/ΔF508	23, female	5936	6.3	92.28
PAO1 1(ATCC 15692)	Burn wound	-	-	5688	6.3	-

Furthermore, minimal passaging of all the PASS strains prior to protein extraction, may likely reduce genetic variations caused due to multiple sub-culturing and may represent closest snapshot of adaptation strategies of the bacteria. In addition, PASS strains displayed considerable diversity in molecular and phenotypic traits including biofilm formation, pigmentation, quorum sensing, motility and adhesion to mucins, thus making them relatively more suitable model organisms to understand the role of *P. aeruginosa* in the CF pathophysiology (138). Nonetheless, strain PAO1 (ATCC 15692), has been used in this study as a control to contrast the molecular features displayed by PASS strains.

1.4.7. *In vitro* mimicking of CF lung nutrient conditions

Pseudomonads are well known for metabolic versatility which is evident by their ubiquitous presence. Specifically, in lungs of CF individuals, *P. aeruginosa* adapts to survive in the heterogeneous CF sputum by utilising a diverse range of carbon sources available while competing with commensal pathogens. CF sputum is nutritionally rich due to the presence of amino acids (139), lipids, DNA and proteins which likely promote the growth of the bacterium (140) evident by frequently reported high colony densities of the bacterium up to 10^8 to 10^{10} CFU/ml (141). The doubling time of *P. aeruginosa* is as short as 40 min when grown in crude CF sputum (142), indicating that the CF sputum provides a robust growth milieu for this bacterium. Several of nutrients in the CF sputum are unusual and often absent in the sputum of the healthy individuals. One such nutrient source is DNA, accumulated in the sputum of the CF individuals originating from lysed host cells, present in concentrations ranging from 5.4 µg/mL to 17.6 mg/mL (143). Increased levels of extracellular proteases in the CF sputum, released from both host and bacteria cleave the proteins into free amino acids. Hence, unsurprisingly, increased levels of amino acids are frequently found in the sputum of the CF affected individuals (5.70 mg/mL) compared to the healthy individuals (2.52 mg/mL) (139). Similar measurements of free amino acids were performed by Palmer *et al* and they found 19 of the 20 standard amino acids in the CF sputum in the range of 4.4 to 24.7 mM. Other micronutrient usually not detected in healthy individuals but found at surprisingly high levels in respiratory tracts of CF patients is iron (242+/-47 ng/mg (144, 145)). However, the majority of it is bound to host chelators (example: ferritin) making it biologically inaccessible for the invading pathogens such as *P. aeruginosa*.

Many bacterial functions are directly or indirectly known to be influenced by nutrient availability including biofilm formation, virulence, motility and toxicity towards host (141). Several studies including proteomic and transcriptomic investigations utilised the knowledge of the nutrient composition of the CF sputum and used crude sputum or attempted to mimic the nutrient conditions through surrogate growth medium in the lab (141, 146-148). One of the initial studies by

Ohman and colleagues (149) used fresh, crude sputum collected from CF and non-CF patients as a growth medium. Their studies concluded one of the commonly observed features of mucoidy of *P. aeruginosa* to be an adaptive modification (149). Further, Palmer *et al* used the crude non-dialysed sputum for the growth of *P. aeruginosa* for transcriptome studies and concluded that the virulence factors are elevated in expression when the bacterium is grown in the sputum (147).

The problems of crude sputum being heterogeneous, complex and difficulty in collecting through expectoration, or extransplanted lungs, made scientists to synthetically mimic the nutrient conditions of the sputum in the lab (141, 150, 151). Sriramulu *et al* (152) utilised a̲rtificial s̲putum m̲edium (ASM), to demonstrate the importance of several nutrients (iron, amino acids, and lecithin) and genes essential for the formation of tight colonies of *P. aeruginosa* thus revealing the mechanistic insights into colonisation of the bacterium in the CF lungs. Palmer and colleagues (141) measured concentrations of the amino acids in the sputum and designed a surrogate medium, s̲ynthetic c̲ystic f̲ibrosis m̲edium (SCFM). They demonstrated the growth pattern of *P. aeruginosa* in SCFM is comparable to the growth in the sputum derived from CF patients. Additionally, they demonstrated similar expression levels of carbon utilisation genes when grown in both SCFM and the sputum (141), indicating the utility of the SCFM in mimicking nutrient conditions *in vitro*.

Unlike other media utilised to mimic CF lung nutrient conditions, SCFM with defined nutrients provides options to adjust the nutrients to evaluate their specific roles in supporting the growth and pathogenicity of the bacterium. For instance, Palmer *et al* through a reductionist approach demonstrated, aromatic amino acids including phenylalanine and tyrosine, play a central role in cueing virulence factor production in the CF lungs (141). A recent follow-up study by the same group reported improvement of the composition of the SCFM by the addition of the macromolecules DNA, lipids, GlcNAc, and mucin to generate a nutrient composition closer to the *in vivo* composition found in sputum (142).

In summary, host-pathogen interplay in CF is intricate and multifactorial. During infection, *P. aeruginosa* undergoes micro-evolution in the host and several factors including, intrinsic resistance to drugs, quorum sensing, flexible genome, virulence factors, and immune evasion strategies confer a survival advantage to the bacterium which makes it a relatively more successful pathogen and tough to eradicate completely. The traditional reductionist approach has generated a wealth of information about mechanism underpinning the molecular pathways of *P. aeruginosa* adaptation; however, system level, holistic "omics" investigations including proteomic analysis provides new opportunities to understand the complex and delicate interaction between host and pathogen. Proteomics in combination with mass spectrometry has been instrumental in driving such understanding. The following section will introduce the field of proteomics and their role in generating system level understanding of the *P. aeruginosa* pathogenesis in CF.

1.5. <u>Proteomics and mass spectrometry</u>

Proteomics refers to the analysis of a set or subset of proteins of an organism expressed in a given time and condition (153). Proteomics broadly involves two aspects: (1) large-scale identification and cataloguing of proteins, and (2) relative or absolute quantification of the proteins. This information can then be interpreted to gain a systems-level understanding of biology. Mass spectrometry (MS) is widely regarded as the "workhorse" of proteomics. Since MS technologies for characterisation of a complex mixture of *intact* proteins is in its infancy, modern proteomic analysis predominantly uses a peptide-centric approach, commonly termed bottom-up proteomics.

Bottom-up proteomics (Figure-1.4A) involves controlled proteolytic digestion of the proteins using enzymes, commonly Lys-C and trypsin. The former cleaves proteins on the C-terminal side of lysine while the latter cleaves at the C-terminal of lysine and arginine amino acids. Given the relative high frequency of Lys and Arg residues in proteins, cleavage with these enzymes generates peptides considered to be of ideal size and charge for high-pressure liquid chromatography (HPLC) coupled positive

electrospray ionization (ESI) mass spectrometry. Under acidic conditions, these peptides usually carry two positive charges (N-terminal amino group and at the C-terminal of lysine or arginine on the side chain), aiding their ionization. Proteolytic peptides are commonly separated by reverse phase (RP) chromatography preferably on an octadecyl chain (C18) particle. Peptides bind on RP based on hydrophobicity and elution is achieved using an increasing concentration of organic solvent. However, for complex biological samples often one-dimensional RP chromatography does not necessarily reduce the sample complexity sufficiently to ensure extensive proteome profiling. Hence, biphasic columns comprising of a combination of RP with a strong cation or anion exchange columns may be employed (e.g. Multidimensional protein identification technology (MudPIT) (154)). Alternatively, off-line systems for peptide fractionation employing a range of phases can be used (e.g. SCX, SAX, high-pH RP, HILIC) (155).

For peptides to be introduced into the MS they must be able to carry a charge. This is commonly achieved by electrospray ionization (ESI) by applying a voltage between the RP column and the MS orifice. ESI being a mild ionization technique generates singly and multiply charged molecules ideal for MS analysis (156). In the last decade, mass spectrometers have evolved tremendously to be rapid in scanning speed, have highly sensitive detection and high mass accuracy (low ppm level), ideal for the analysis of a complex mixture of peptides. Orbitrap™ and Q-TOF are widely used instrument architectures for proteomic analysis as these provide fast, high accuracy mass measurements.

A
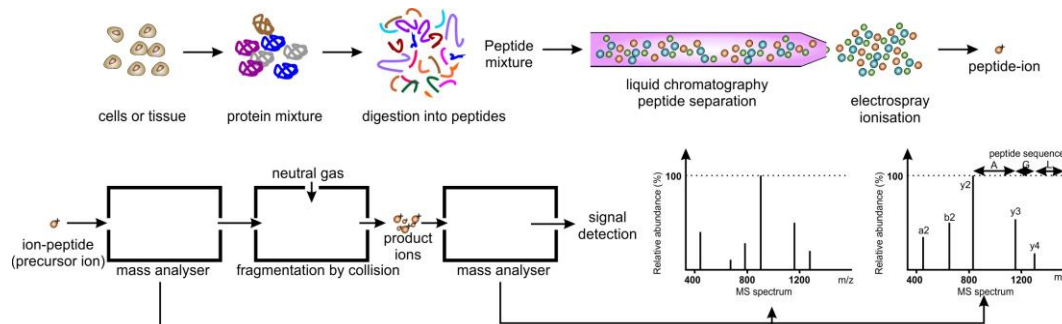
B
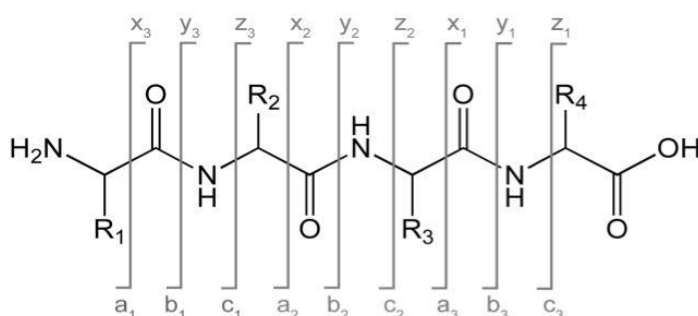
Figure-1.4: An illustration of a typical bottom-up mass spectrometry analysis of proteolytic peptides. (A) Cellular proteins are proteolytically digested, separated by chromatography, subsequently charged and ionized via ESI. For sequencing the amino acid backbone, the peptides are further broken by colliding with an inert gas. The mass spectrums represent the results of this analysis: the distribution of ions in the sample by their mass-to-charge ratio. (B) The types of peptide fragment ions observed in an MS/MS spectrum. Peptide fragments will only be detected if they carry at least one charge. If the charge is retained on the C-terminal, the ion type is either *x, y* or *z*. On the other hand, if the charge is retained on the N-terminal fragment, the ion is termed as either *a, b* or *c*.

Having measured the mass-to-charge ratio (m/z) and intensities of peptide ions eluting from RP chromatography, the MS can be instructed to generate primary sequence information using tandem-MS, also termed MS/MS or MS2, in which, a specific peptide ion is isolated and energy is imparted by collision with an inert gas (such as argon, helium or even nitrogen) to break the peptide at defined bonds. A variety of dissociation techniques can be used, a common one being collision induced dissociation (CID), which fragments the amide bonds between amino acids resulting in *b-ions* (charge retained by the amino-terminal peptide fragment) and *y-ions* (charge retained by the carboxy-terminal peptide fragment) (Figure-1.4B). Quadrupole instruments predominantly generate more intense *y-ions*, whereas ion trap mass spectrometers generate both *b-* and *y-ions* at similar intensities, but suffer from the

one-third cut-off rule which limits the lower mass limit for product ion detection. Recently, higher energy collisional dissociation (HCD) fragmentation (available with Orbitrap instruments) has been described, which produces peptide fragment ions similar to these seen with Q-TOF CID (157). In contrast to traditional ion trap-based CID fragmentation, HCD fragmentation has no low-mass cut-off, yielding higher quality MS/MS spectra (157).

Large scale proteomic data comprising of millions of spectra has outpaced our ability to interpret each spectra manually. Hence, sequence assignment is invariably done using computer algorithms such as MASCOT (158), SEQUEST(159), ProteinPilot (with Paragon algorithm)(160), among many others. These search algorithms match the empirical MS/MS spectrum with *in-silico*, theoretical fragment ion information generated from known protein (target) databases and assign a probabilistic matching score. The greater the matching ion features between the empirical and theoretical mass spectrum, the more confident the identification match and subsequently assigned score. Post-search, false discovery rate (FDR) analysis is estimated routinely through a target-decoy search strategy, where empirical data is compared against a decoy database (reverse sequence) where there are no true matches. The number of observed matches in this search provides an estimate of the false matches in the results from the target database (161). Based on search algorithm score and FDR threshold, peptides are selected to make the final list of proteins. Up to 1% FDR threshold at the peptide level is considered standard in the field.

1.5.1. Data Dependent (DDA) mass spectrometry and its applications

In a typical LC-MS analysis, peptides eluting from the chromatographic column are predominantly analysed by MS in two ways. The first and most widely used method, Data-Dependent Acquisition (DDA) or Information-Dependent Acquisition (IDA) and the second method, recently gaining wide attention is Data Independent Acquisition-MS (DIA-MS). DDA-MS provides automated and high throughput capabilities to analyse complex proteomes. In this method, during peptide elution, first, a MS1 survey scan is performed to record intact peptide m/z, charge and

intensities of ions at a given time. In the second step, an automated, "on-the-fly" selection criterion is generated depending on the "Data/Information" from the MS1 survey scan, (predominantly intensity and charge state), and used to perform a MS2 scan on "N" most intense ions of a given charge state. The cycle of MS1 and concomitant MS2 is repeated for the entire chromatographic elution gradient, with the duty cycle speed defining the number of ions selected for fragmentation and MS2 analysis in any given cycle (162). Although DDA is a method of choice for identification of a maximal number of proteins from samples, the process of ion selection for tandem MS is stochastic and suffers from run to run variability, dynamic range issues, and under-sampling. Replicate analysis is often needed to ensure high sample coverage. Recent advancements in workflows try to address this issue by reducing the complexity of the samples prior to MS analysis by orthogonal, off and on-line fractionation of samples using MudPIT (163), High pH basic reverse phase separation (164), among others as discussed earlier. Additionally, by increasing C18 column length (30-50 cm) and run time (3h gradient time) greater resolution can be achieved leading to increased proteome coverage (165).

DDA mass spectrometry is not only used for identification of the proteins but also for accurate, relative or absolute quantification of the proteins. One of the widely used quantitative proteomics scheme using DDA-MS has been isobaric chemical labelling of the peptides using the Isobaric tag for relative and absolute quantitation (iTRAQ™) (SCIEX) (166) and tandem mass tags (TMT™) (Thermo Fisher Scientific) (167) reagents. These reagents contain a reporter group/mass tag, balancer/mass normaliser group, and a peptide reactive/amine reactive group which react with primary amines in the side chain amines of amino acids and N-terminus of the peptide (Figure-1.5A-B). The reporter group with various masses is generated by a combination of isotopic elements, thus allowing multiplexing ranging from 2-plex to 10-plex. Concomitantly, the mass of the respective balancer tag also varies, ultimately the sum of the mass of the balance and reporter group remains constant. Identical peptides originating from different samples are labelled with different versions of TMT/iTRAQ tags. Although peptides are labelled they remain "isobaric" during MS1

scan, the low molecular weight reporter mass tags are liberated by gas phase collision fragmentation and detected in MS2. Intensities of reporter ions are used for relative quantification of peptides originating from different samples (Figure-1.5C). Isobaric labelling based quantitation is advantageous because: 1) labelling does not increase the complexity of the sample separation in LC, 2) enables multiplexed quantification of up to 10 samples increasing the throughput by allowing parallelization of sample quantification, 3) complexity of MS1 mass spectra is not increased since the peptides are isobaric in nature, 4) labelling is applicable to any biological system and does not require actively synthesised proteins.

A commonly reported problem associated with the isobaric labelling is pervasive, distortion of quantitative accuracy due to interference caused by near isobaric ions in the sample which are readily co-isolated and co-fragmented leading to suppression of reporter ions and quantitative under-estimation. Hass and Gygi laboratories published methods (168, 169) to mitigate this problem using additional gas-phase manipulations by MS3 scan. In this method, multiple intense TMT/iTRAQ labeled MS2 fragment ions are co-isolated using isolation waveforms with multiple notches and co-fragmented using CID or HCD to generate MS3 spectrum (MultiNotch MS3) with reporter ion intensity based quantitative data that are free from the background and leads to accurate quantification. Nonetheless, this additional round of MS results in ion loss, so lacks the sensitivity of conventional analysis using MS2.
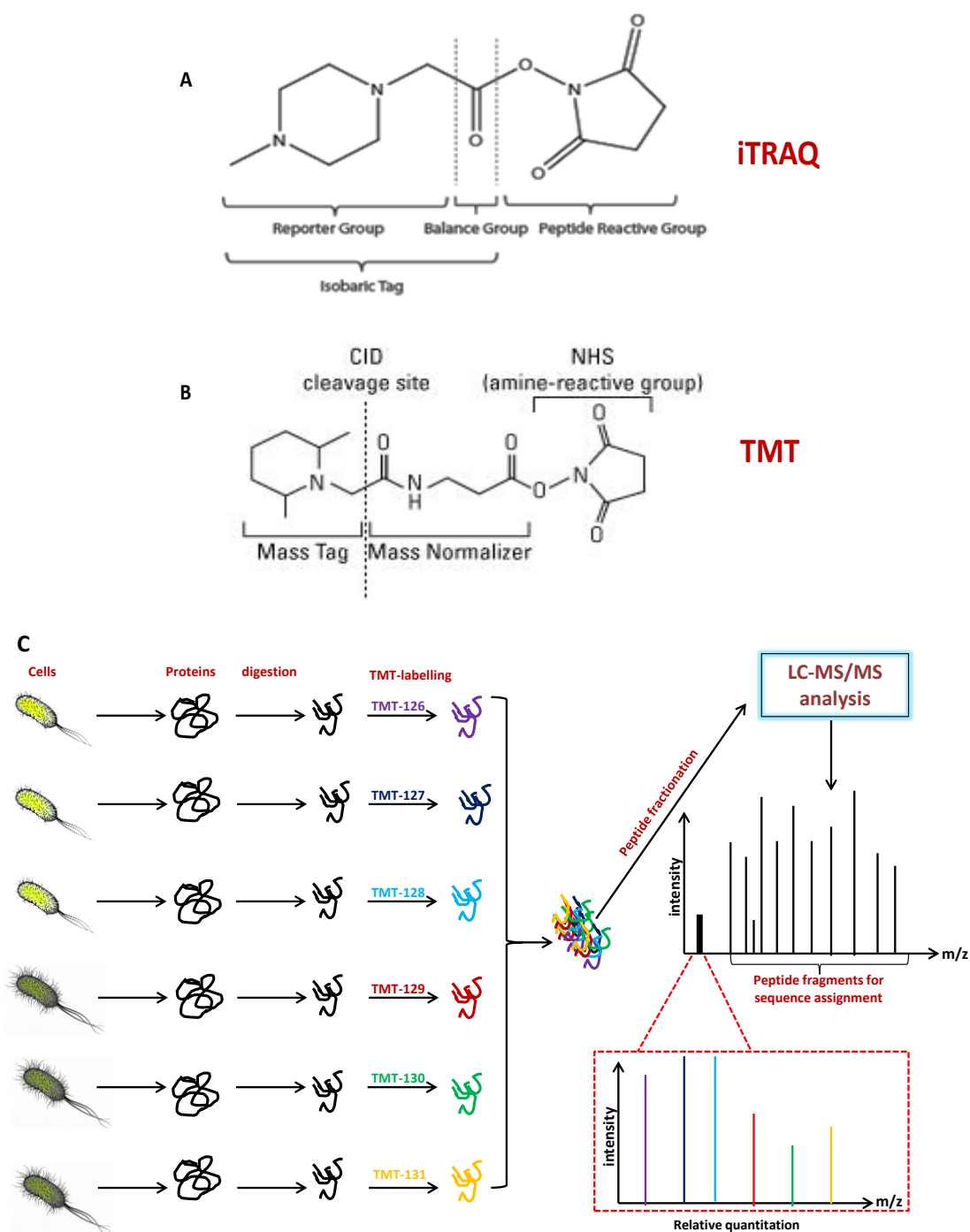
Figure-1.5: Isobaric labelling based multiplexing quantitative proteomics. Isobaric labelling consists of chemical derivatization of peptides using iTRAQ (A) and TMT (B) reagents that incorporate mass tags into peptide samples. (C) Illustration of multiplexing based proteomic quantitation. Peptide samples are labelled with individual mass tags and then pooled for LC-MS/MS analysis. Since the masses of all the isobaric tags are the same, peptides with similar mass originating from different samples co-elute and are analysed by MS. After tag cleavage during gas phase fragmentation and MS2 scan, the tag intensities are used for quantification, while, the peptide fragment ions are used for assigning sequence of the peptide for identification.

1.5.2. <u>Data Independent Acquisition (DIA) mass spectrometry and its applications</u>.

DIA-MS (also known to as MS/MS ALL) does not rely on data acquired during the MS1 scan, instead, all the ions detected in a MS1 survey scan are selected for fragmentation followed by MS2. One of the best developed DIA methods, <u>S</u>equential-<u>W</u>indow <u>A</u>cquisition of all <u>TH</u>eoretical mass spectra (SWATH-MS) (170), is known as SWATH-MS, combines quantitative accuracy and reproducibility of targeted MS (i.e. selected reaction monitoring-MS) and allows large-scale identification of proteins in complex proteomes. In SWATH-MS, quantitation is based on XICs from MS2 scans. As several product ions can be used for quantitation, excellent selectivity and accuracy is produced. Moreover, since ion selection in DIA for tandem MS is not biased, this technique shows excellent run to run reproducibility for ion detection.

As illustrated in Figure-1.6, the method can be explained in two steps. In the first step, all the ions in a sequential window defined by the user (example, ~25 Th windows in m/z 400-1200 range) are fragmented recursively through the entire liquid chromatography (LC) separation, resulting in a complex array of information containing retention time, m/z and intensity information for each precursor selection window. Of note, to empower SWATH-MS, mass spectrometer must have a rapid scanning speed to cover adequate data points across a typical chromatographic peak to reconstruct ion-chromatogram with sufficient number of data points to gain reliable quantification. It is estimated that, Quadrupole-Time of flight (TripleTOF 5600, Sciex), instrument used for acquisition of the data in this thesis, is capable of stepping through typical ~25 Th windows repeatedly with accumulation time of 100 ms and 1 Th window overlap across m/z 400-1200 range, in duty cycle of ~3.2 seconds (170). This cycling time is adequate to generate a 30 s wide chromatographic peak for each analyte for accurate and reproducible quantification.

In the second step of the SWATH-MS analysis workflow, resulting transition ion information, using a software algorithm such as OpenSWATH (171), PeakView™

(SCIEX) is matched to a pre-made peptide spectral library to generate peptide/protein identification and quantification results. Of note, SWATH-MS currently relies on ion library generated in a DDA mode, which underscores the necessity of having a high-quality spectral library. If the peptide is not registered in the spectral library it cannot be identified and quantified in SWATH-MS.
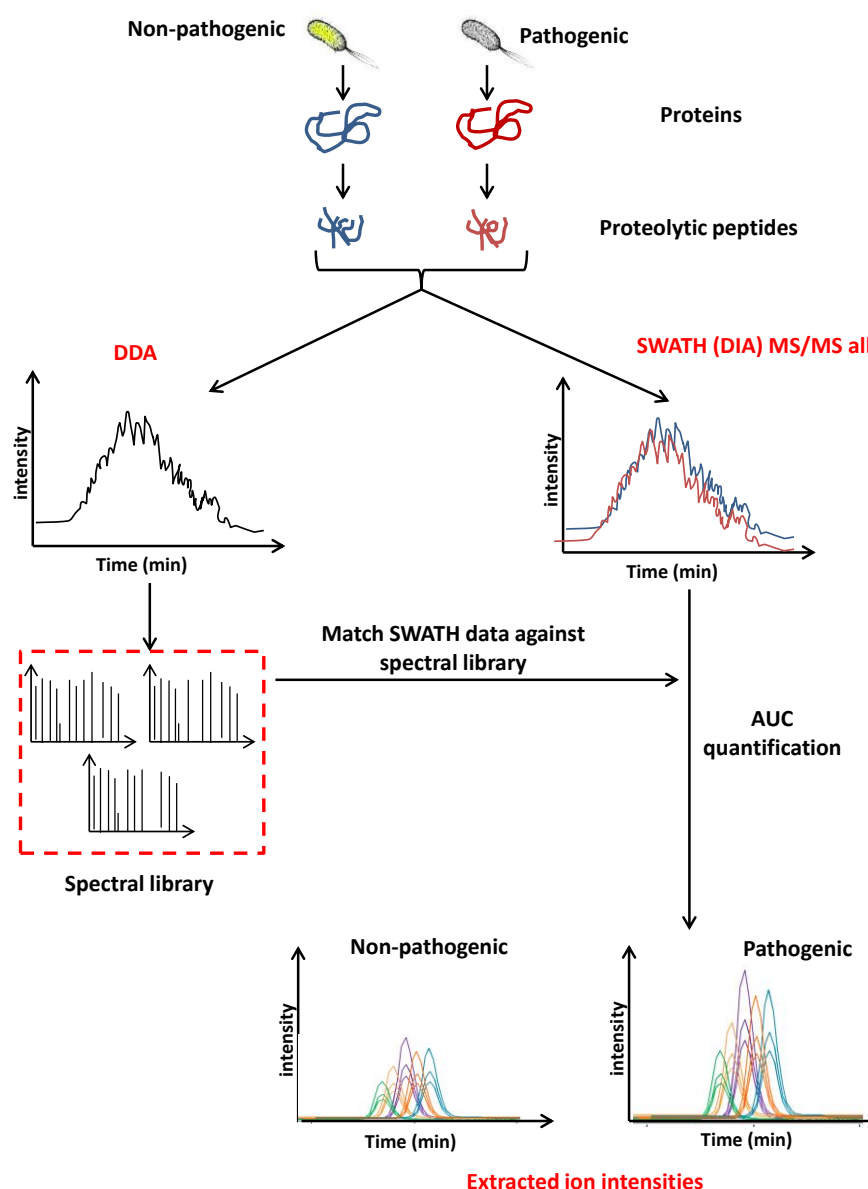
Figure-1.6: Illustration of SWATH-MS proteomic analysis. As the first step of analysis workflow, a high-quality spectral library is constructed by analysing proteolytic peptides using DDA-MS. The same sets of samples are individually run by SWATH-MS (MS/MS all) workflow Acquired mass spectrums are searched against the spectral library for identification. Extracted ion chromatogram is reconstructed for each identified peptide. The area under curve information is used for quantitative comparison of protein quantities across the samples.

1.6. <u>Research context and aims:</u>

The research described in this thesis was carried out over 3.5 years in the context of a larger cross-discipline research project at Macquarie University to investigate microbial interaction networks associated with CF. As part of the project, four *P. aeruginosa* strains (PASS1-4) were isolated from the sputum of cystic fibrosis patients and the genomes were sequenced by my colleagues (138). These strains were also subjected to various phenotypic analyses showing differences in colony morphology, pigmentation, carbon source utilisation, biofilm formation and virulence (138).

To augment this knowledge, I conducted proteomic studies of *P. aeruginosa* PASS strains with an aim of furthering our understanding of how cellular proteins may contribute to *P. aeruginosa* adaption to the harsh microenvironment of the CF lung. This approach would allow mechanistic insight to better understand how *P. aeruginosa* is a successful coloniser in the lungs of CF-affected individuals. To contrast with the characteristics of these clinical isolates, the commonly used laboratory strain PAO1 was also studied.

Specific aims were as follows:

1) Determine quantitative global proteome level similarities and differences between *P. aeruginosa* PAO1 and four novel CF isolates (PASS1-4) cultured in laboratory broth medium.

2) To create a membrane sub-proteome profile of *P. aeruginosa* CF strains (PASS1-3) cultured in CF lung nutrient mimicking conditions, using mass spectrometry and quantitatively compare with PAO1, to determine CF specific membrane proteome modulations.

3) Investigate the response of the *P. aeruginosa* proteome to oxygen limitation stress under CF lung nutrient mimicking conditions.

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Proteomics of host and pathogens in cystic fibrosis

Rationale:

This chapter consists of a published review article;

Proteomics of hosts and pathogens in cystic fibrosis. <u>Karthik Shantharam Kamath</u>*, Sheemal Shanista Kumar, Jashanpreet Kaur, Vignesh Venkatakrishnan, Ian T Paulsen, Helena Nevalainen and Mark P Molloy. *Proteomics Clin Appl*. 2015 Feb;9(1-2):134-46.

The review article explains the role of proteomic techniques in generating systems level understanding of cystic fibrosis biology from a host and pathogen perspective.

Contribution:

Sheemal and Jashan contributed in the section 1 and section 3.2 respectively. Rest of the sections of the review were written by me. Prof. Mark Molloy, Prof. Helena Nevalainen and Prof. Ian Paulsen read and approved the manuscript.

REVIEW

Proteomics of hosts and pathogens in cystic fibrosis

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Cystic fibrosis (CF) is a congenital disease that results in great morbidity and mortality mainly in the Caucasian population. Although CF is a monogenic disease caused by mutation in the CF conductance transmembrane regulator (CFTR) gene, most of the related mortality can be attributed to infection mediated by opportunistic bacterial and fungal pathogens. Over the past decade, advancements in the field of proteomics have helped to gain insight into the repertoire of host and pathogen proteins involved in CF pathophysiology. This review provides an overview of the contributions of proteomic studies in advancing our knowledge of the biology of CF and disease progression associated with pathogen infection and host defense responses.

☐ Additional supporting information may be found in the online version of this article at the publisher's web-site

1   Introduction

Cystic fibrosis (CF) is an autosomal recessive congenital disorder affecting one in 3500 live births, with the highest prevalence among Caucasian populations [1]. This disease was first described in 1930, and was mainly characterized by malabsorption of fat and protein, congenital steatorrhea, growth failure, and bronchopulmonary infections in affected individuals [2]. However, the major reason for mortality in CF patients was identified through progression of lung disease caused by chronic respiratory infection associated with microorganisms and systemic host-driven inflammation. Only later in 1989, the genetic basis of CF was revealed with the discovery of a loss-of-function mutation in the CFTR (cystic fibrosis transmembrane conductance regulator) gene encoding the transporter channel for chloride and bicarbonate ions

in epithelial cells, airways, sweat, and pancreatic ducts [3]. To date, over a thousand different mutations have been identified in the CFTR gene, however, deletion of the codon for phenylalanine at position 508 (ΔF508) accounts for approximately 70% of all CF patients. CFTR mutation causes a defect in water transport in and out of cells, resulting in excessive secretion of abnormally thick and viscous mucus that is most detrimental to normal lung respiratory function [4, 5].

Defective mucociliary clearance of thick stagnant mucus in the lung airways of CF patients leads to infection and inflammation, which together acts to further deteriorate lung function. The relationship between infection and inflammation in a disease like CF is complex, particularly in regard to cause and effect mechanisms. However, it is evident that failed clearance of dehydrated mucus, which provides a habitat for opportunistic pathogens, promotes host neutrophil invasion with subsequent release of oxidants and proteases that cause progressive damage to lung tissue in a self-perpetuating cycle [6, 7] (Fig. 1).

A diverse range of microorganisms including bacteria, fungi, and viruses have been isolated from lungs of CF patients. A detailed list of pathogens commonly associated with CF is summarized in Supporting Information Table 1. The most commonly identified bacteria colonizing the CF airway include *Pseudomonas aeruginosa, Burkholderia cepacia* complex (BCC), *Staphylococcus aureus,* and *Haemophilus influenzae* [8]. Among these pathogens, *S. aureus* and *H. influenzae* are the most commonly isolated bacteria from the respiratory tracts of CF patients in early stages of infection [9]. However,

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Abbreviations: AHL, acyl-homoserine lactone; **BALF**, bronchoalveolar lavage fluid; **BCC**, *Burkholderia cepacia* complex; **CF**, cystic fibrosis; **CFTR**, cystic fibrosis transmembrane conductance regulator; **IL**, interleukin; **LPS**, lipopolysaccharide; **PQS**, phenazine-mediated QS; **QS**, quorum sensing; **T2SS**, type II secretion system; **TCA**, tricarboxylic acid; **UPR**, unfolded protein response

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Proteomics Clin. Appl. 2015, *9*, 134–146   135
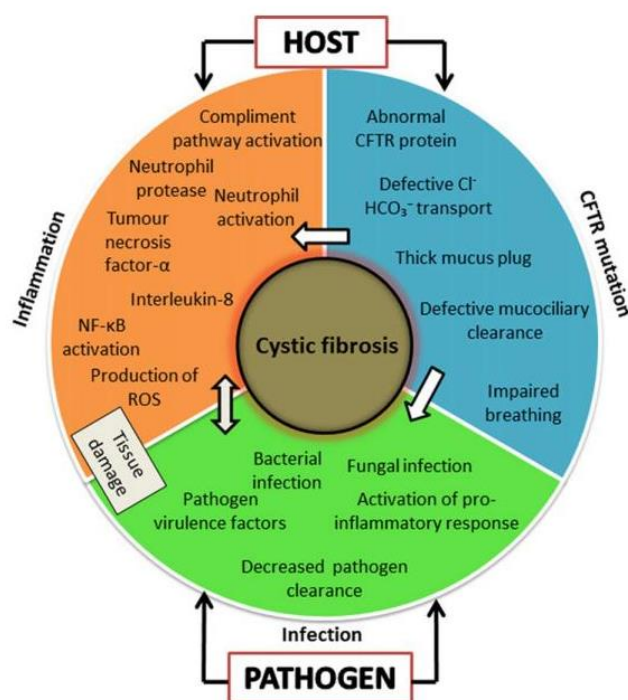
Figure 1. Pathophysiology of cystic fibrosis is a collective result of defective CFTR, infection, and inflammation. Both the host inflammatory response and bacterial virulence factors result in progressive lung tissue damage.

longitudinal studies show the CF lung microbiome changes with time, with decreased species diversity and dominance of *Pseudomonadaceae* members in more mature patients [10]. The main colonizing bacterial pathogens display remarkable ability to escape multiple classes of antimicrobial agents due to classical lateral exchange of drug resistance genes, mucoidy conversion, and biofilm formation, among other strategies [11]. In addition to bacteria, lungs of CF patients are often invaded by yeasts and filamentous fungi [12].

Considerable research effort has been invested to explore the relationship between CF and pathogen infection using proteomic analysis of CF patient tissues and biological fluids, and a variety of bacterial and fungal pathogens. In this review, we have examined the various proteomic approaches and key contributions of these studies to better understand the interaction of hosts and pathogens in the setting of CF disease.

2 Proteomics of CF host biofluids and tissues

In the past decade, proteomics has been used to investigate a variety of CF host tissues and biofluids. These include experiments conducted with serum/plasma, lung/nasal epithelial tissues, sputum, bronchoalveolar lavage fluid (BALF), and nasal cell linings.

2.1 CF host biofluids

Unlike tissue samples, which need specialized retrieval, procedures such as biopsies, serum, and sputum can be readily and repeatedly collected intravenously and through expectoration, respectively. Nasal cell scrapings can also be obtained in a minimally invasive manner. BALF extractions have been widely reported for collection of samples for the proteomic analysis of CF patients [13, 14].

2.1.1 Proteomics of BALF

BALF enables sampling of the lower extraluminar space of the pulmonary airways. This sampling technique can provide an indication of lung function and mucosal clearance since it retrieves a variety of components including exuded plasma, dead tissue, pathogens, and inflammatory molecules. von Bredow and colleagues used 2DE to compare the composition of the BALF proteome of CF patients and healthy controls to assess the degree of proteolytic degradation of surfactant protein A; an important innate host defense component of the lungs [15]. They demonstrated significant proteolysis of surfactant protein A and other BALF recovered proteins in CF patients, while BALFs from healthy individuals did not show similar protein degradation patterns. The observed degradation was the result of elevated levels of active and abundant neutrophil-derived proteases in CF patients. This key study

provided one of the initial clues supporting neutrophil dominant inflammation accompanied with impaired pathogen clearance [15]. In a follow-up study by others using a larger set of samples, analysis with SELDI-TOF-MS confirmed the findings related to increased levels of neutrophil-associated proteins in children affected with CF [13]. Using a CF mouse model, Zahm and co-workers, showed elevated levels of interleukin 8 (IL-8) and neutrophils in BALF samples confirming an active, inflammatory response [16]. Gharib and colleagues used shotgun proteomics and conducted an extensive LC-MS/MS study on the BALF proteome of CF and non-CF individuals using label-free spectral counting quantification [17]. Using this approach, they identified 896 distinct proteins in BALF mainly in relation to oxidative stress and aberrant functioning of airway and innate immunity responses. Given the extensive protein identification delivered through the gel-free approach, many of the reported differences had not been previously demonstrated [13, 15, 17]. Therefore, this paper provided a useful demonstration of deeper biological understanding that could be obtained by transitioning from 2DE-based studies to LC-MS/MS analyses.

2.1.2 Proteomics of sputum and serum

In comparison to BALF, CF patient sputum is less well characterized, which can be attributed to the difficulty in sample preparation and analysis due to the high abundance of heavily glycosylated mucin proteins [18]. Other abundant serine and metalloproteases, immunoglobulins, and serum albumin can also mask less-abundant proteins in sputum, thus hindering the analytical capability of traditional proteomic methods.

Pedersen et al. described screening for potential antigenic biomarkers in the sputum and serum collected from CF patients with bacterial infections [19]. The CF patients showed increased autoimmunoreactivity toward *Pseudomonas* proteins, stress response proteins, immunosuppressive proteins, and proteins of the alginate synthetase pathway [19]. Further 2DE-based profiling of CF sputum revealed elevated levels of immune reactive molecules such as IgGγ heavy chain fragments, IL-8, and myeloperoxidase [20]. Elevated concentrations of pro-inflammatory cytokines such as tumor necrosis factor α and ILs (IL-6 and IL-8) were observed in sputum of CF children compared to children with bronchiectasis and no CF [21]. Thus, sputum obtained from CF patients has been regularly monitored in hospitals to assess the inflammation level and infection status in the CF airway.

The proteomic analyses of CF patient serum have mainly focused on seeking a diagnostic biomarker. Reverse capture protein microarrays have been utilized to screen differences in protein expression in sera between CF and non-CF patients. This study showed that mediators of inflammation, such as the NF_KB signaling pathway, were significantly elevated in CF patients [22]. Serum analysis using complementary 2DE and LC–MS/MS techniques revealed differentially expressed proteins in relation to dysfunction of innate

immune system, chronic inflammation, protease/antiprotease imbalance, aberrant airway remodeling, and nutritional imbalance [23]. A proteomic screen of sera from CF patients with various stages of infection showed that the *P. aeruginosa* outer membrane protein L (a nontype-3 secretion system) could be used as a diagnostic marker for an early-stage *P. aeruginosa* infection [24].

2.2 Cellular effect of CTFR mutation investigated in CF models

Proteomic studies have been used with various cell model systems to gain a molecular understanding of the ΔF508 CFTR mutation. To study the effect of this mutation, Davezac et al. transfected HeLa cells, which lack endogenous expression of CFTR, with the wild-type CFTR gene or CFTR DNA carrying the ΔF508 mutation and used 2DE to compare global protein expression profiles [25]. They reported that cytokeratins 8 and 18 showed varied expression implicating a role for these structural cytoplasmic proteins in the trafficking of the CFTR protein to the plasma membrane [25]. Two-dimensional gel electrophoresis (2DE) profiling of CF bronchial epithelial cells (IB3-1) revealed alterations in proteins associated with inflammation, chaperone, and proteasome proteins, phosphatase inhibiting proteins, and calcium signaling [26, 27]. Further proteome screens of IB3-1 cells treated with the candidate drug 4-phenylbutyrate demonstrated reversal of the CF phenotype by regulation of Hsp70, which aids in the folding of CFTR [28]. These findings were validated in a follow-up study using immunoprecipitation, where protein interactors of CFTR obtained after chemical rescue or correction of mutation were analyzed using 2DE and MS. These results strongly implicated the requirement of active Hsp70 family proteins to facilitate CFTR folding and membrane insertion [29]. In further agreement, Wang and colleagues used MudPIT MS to investigate interacting partners of CFTR [30]. Their study showed strong correlation between the CFTR protein and chaperone Hsp-90 and co-chaperone Aha1. Aha1 in association with Hsp-90 was shown to affect ER clearance of the protein [30]. Di-arginine (RXR) motifs in proteins play a key role in retaining improperly folded proteins in the ER. Gomes-Alves and colleagues studied the effects of RXR motifs on ΔF508-CFTR protein maturation [31]. The RXR motifs (ΔF508/4RK-CFTR) were inactivated and using 2DE they compared the protein expression profiles between wild-type CFTR, ΔF508del-CFTR, or ΔF508/4RK-CFTR. ΔF508/4RK-CFTR showed stronger unfolded protein response (UPR) and also downregulation of proteasome machinery necessary for CFTR degradation [31]. This substantiates some earlier work, which suggested overexpression of ΔF508del CFTR is strongly related to ER stress and UPR activation [32]. Together these studies suggest that RXR motif could be used as a drug target for correction of the effect of ΔF508 mutation. A recent study used MudPIT to generate a comprehensive global proteome map of

ΔF508-CFTR mutated lung epithelial cells. This study reported over 5000 proteins with roles in protein folding, UPR, endocytosis, proteolysis, and ubiquitin-mediated degradation of deformed proteins, thereby providing a comprehensive proteomic baseline model for CF researchers [33]. These studies reveal key proteins involved in generation of functional CFTR proteins and also suggest potential drug targets to counter the ΔF508 mutation.

To summarize through this section of the review, proteomic analysis of CF patient biofluids, particularly BALFs and sputum, has revealed the active role of host neutrophils in contributing to the pro-inflammatory environment of the CF lung. Analysis of patient serum has been mostly limited to diagnostic applications. The cellular effect of the major CFTR mutation ΔF508 has been extensively characterized by 2DE and LC-MS/MS showing an important contribution of chaperones and the UPR pathway in processing of the CFTR protein.

3 Proteomics of microorganisms associated with CF

The abnormal extracellular environment of CF lungs provides an ideal habitat for opportunistic microbial pathogens. Hence, infections are considered as a major complication arising in CF disease ultimately leading to mortality. Pathogen invasion/infection not only causes direct harm to the host cells through secretion of virulence factors, but also elicits the host immune response, which leads to inflammation, and ultimately, destruction of lung cellular infrastructure. Pathogens also develop strategies to escape host immune responses and antimicrobial drugs. Lungs of CF patients are often shown to harbor polymicrobial communities, encompassing bacterial *(e.g. Pseudomonas* spp., *Staphylococcus* spp., *Burkholderia* spp.*)* and fungal pathogens (e.g. the yeast *Candida albicans* and filamentous fungus *Aspergillus* spp.). Next-generation genome sequencing technologies have demonstrated the presence of other "emerging" pathogenic species associated with CF (Supporting Information Table 1).

Proteomics has been used as a key tool to study pathogens isolated from the lungs of CF patients as they provide relevant material to investigate features such as modes of invasion, quorum sensing (QS), biofilm formation, cellular communication, and the development of drug resistance. This section focuses on the proteomic studies conducted on microbial pathogens infecting CF lungs and summarized in Supporting Information Table 2.

3.1 Infection by bacterial pathogens

Understanding the bacterial ecology in the lung is of considerable importance in CF as chronic bacterial infection is one of the main causes of respiratory damage [34]. The prevalence of bacterial pathogens in respiratory infections changes with patient age. For example, the major lung colonizer in children with CF is *S. aureus* and in the adult CF lung, *P. aeruginosa* [35]. Here, we have briefly reviewed the key literature findings that have led to a better understanding of infection pathobiology associated with CF.

3.1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, nosocomial, biofilm-forming bacterium commonly present in CF lungs. The success of *P. aeruginosa* as a versatile pathogen can be attributed to its capacity to utilize multiple compounds as energy sources, genomic plasticity, large array of virulence and adhesion factors, capacity to out-compete other pathogens for trace elements, all of which collectively contribute to successful colonization [36, 37]. It causes chronic infection in over 80% of adult CF patients, which strongly correlates with mortality [38]. In CF, these *P. aeruginosa* infections are life threatening and have long remained a treatment challenge due to continuous evolution of drug resistance. The *P. aeruginosa* genome is comprised of approximately 5500 ORFs and about 40% of the genes are annotated as hypothetical/putative demonstrating the need for proteomic studies to improve understanding of the complementing proteome (http://www.pseudomonas.com/). The genomes of *P. aeruginosa* strains most widely used for proteomic studies, PAO1 (laboratory-associated burn wound isolate), PA14 (virulent wound isolate), and AES-1R (Australian epidemic strain, transmissible CF clinical isolate), have been sequenced and annotated [36, 39, 40]. Many proteomic studies have been conducted in relation to infections caused by *P. aeruginosa* and reviewed elsewhere [41]. Here, we briefly review key studies and discuss more recent information.

3.1.1.1 Investigation of cellular and secreted proteins

One of the first proteome studies on *P. aeruginosa* isolated from a CF patient compared the proteome of a nonmucoid and a mucoid strain obtained from the same patient. Using 2DE and MS, ten and four proteins were identified from nonmucoid and mucoid strains, respectively, in an age prior to the availability of the organism's genome sequence [42]. The identified proteins were some of the most abundant cellular proteins representing protein synthesis (50S ribosomal protein L25, 30S ribosomal protein S1, DnaK, etc.), outer membrane structure (OprH, OprF), and resistance to oxidative damage (superoxide dismutase, catalase, alkylhydrogenperoxide reductase). Nouwens et al. created the first membrane proteome reference map using 2DE, which identified 189 proteins, thereby demonstrating the expression of many proteins for the first time [43]. More recently, through the application of 2D chromatography and MS coupled with iTRAQ tagging, approximately one-third of the predicted proteome (1788 proteins) was quantitated. The authors compared the

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expression of proteins among the three strains PAO1, PA14, and AES-1R [44]. This study showed that proteins involved in the acquisition of iron, lipopolysaccharide (LPS) biosynthesis, and antibiotic resistance were elevated in the transmissible clinical strain AES-1R compared to the burn wound isolate/control strain PAO1 and virulent clinical strain PA14. These results suggest that the transmissible strains have specialized metabolic adaptation strategies, which provides them a selective advantage during early stages of infection. The findings may help toward the discovery of better therapeutics to address problems of transmissibility and acute infections caused by *P. aeruginosa* in CF.

Bergamini and colleagues investigated the effect of secreted proteins derived from *P. aeruginosa* PAO1 and an acute and a chronic clinical isolate (isolates from CF patient collected in the gap of 7.5 years) grown under microaerobic and aerobic conditions on epithelial CF cells [45]. They observed that the mRNA expression levels of a key pro-inflammatory cytokine IL-8 were increased after exposure of the cells to *P. aeruginosa* secreted proteins. IL-8 is known to activate neutrophils via binding to chemokine receptor CXCR1. The parallel MudPIT MS profiling of PAO1 and clinical strains of *P. aeruginosa* identified elevated levels of bacterial matrix metalloproteases [45]. Further, to establish a connection between increased amounts of bacterial matrix metalloproteases and CXCR1 cleavage, the authors treated peripheral blood neutrophils isolated from healthy donors with secreted proteins from both chronic and acute clinical isolates of *P. aeruginosa*. A significant decrease in CXCR1 was observed when exposed to proteases derived from acute strains compared with chronic strains [46]. This observation opens up the possibility of involvement of microbial proteases in the modification of host phagocytosis, supporting more efficient pathogen colonization [45].

3.1.1.2 Investigation of response to stress

Expression of certain *P. aeruginosa* genes is governed by environmental factors such as the availability of nutrients and oxygen. For instance, limited availability of magnesium triggers expression of virulence factors such as CF-specific LPS at the cell surface, which confers resistance to antimicrobial peptides. LPS further activates the pro-inflammatory response mediated through the host Toll-like receptor 4 [47]. A study using MS and ICAT quantification suggested triggering expression of proteins in relation to virulence and QS under conditions of magnesium stress. Specifically, proteins involved in phenazine-mediated QS (PQS) were shown to be increased fivefold under conditions of magnesium limitation. TLC of PQS from wild-type PAO-1 and a PQS-null mutant confirmed these results [47, 48]. *Pseudomonas aeruginosa* is known to colonize the host under hypoxic or anaerobic conditions with increased tolerance to host immune system responses and antimicrobial drugs [49]. The adaptation into anaerobic conditions is supported by switching to alternative terminal electron acceptors, such as nitrous oxide

(N$_2$O), nitrate (NO$_3^-$), nitrite (NO$_2^-$), or arginine [50]. Platt et al. investigated anaerobic NO$_3^-$ and NO$_2^-$ respiration of *P. aeruginosa* using transcriptomic and proteomic approaches [50]. The authors found that the genes associated with denitrification were significantly upregulated under anaerobic conditions. Further, these authors observed increased expression of proteins involved in detoxification of toxic nitric oxide (NO) that was produced during anaerobic respiration [50]. This suggests that the *P. aeruginosa* has the genetic flexibility to adapt to a changing and harsh microenvironment such as that developed during hypoxia.

3.1.1.3 Proteomic studies in lung-mimicking conditions

Some recent proteomic studies have attempted to investigate protein expression in *P. aeruginosa* under growth conditions that mimic the lungs of CF patients. For this purpose, an artificial sputum-mimicking medium, containing components present in the mucus such as mucin, free DNA, and amino acids, has been used. It has been shown that mucin is one of the key factors for microcolony formation of *P. aeruginosa* [51]. Hare et al. compared the whole cellular proteome of *P. aeruginosa* strains, PAO1 and AES1-R, and concluded that iron acquisition plays a major role in the adaptation of CF-associated *P. aeruginosa* to the lung-mimicking environment [52]. Similar specialized iron uptake systems utilizing siderophore receptors have been shown in another CF-associated Gram-negative bacterium, *Burkholderia* spp. [53]. Proteomic analysis of secreted proteins of *P. aeruginosa* AES-1R strain grown in artificial sputum media led to the identification of 57 proteins by 2D-LC-MS/MS including the protease PasP and enzymes associated with QS such as LasB, LasA, and chitin-binding protein (CbpD). SRM MS was then used for targeted quantification and validation of the expression of these proteins. The study, being the first to investigate the *P. aeruginosa* secretome in a CF lung-mimicking culture medium, revealed several proteins that play key roles in establishing the infection and mediating inflammation [54]. These studies identified several proteins that were not found in previous studies involving growth on other types of media, thus emphasizing the importance of the model system and growth conditions to study disease-specific protein expression of CF pathogens.

3.1.1.4 Understanding QS and biofilms

QS is a cell density dependent bacterial signaling system used to regulate population. QS signaling molecules regulate many genes that govern complex traits such as biofilm formation swarming motility and expression of virulence factors [55]. Well-characterized *P. aeruginosa* QS pathways are *las*, *rhl*, and *pqs* pathways. In *P. aeruginosa*, both *las* and *rhl* pathways produce QS signaling molecules, acyl-homoserine lactones (AHLs) that are secreted and received by neighboring cells. Both *las* and *rhl* systems are known to be interdependent and act on a feedback loop mechanism. [55]. To study

the network of proteins controlled by the *las* and *rhl* systems, Arevalo-Ferro and co-workers used a isogenic *lasI rhlI* double mutant *P. aeruginosa* PAO1 strain and selectively focused on proteins that were associated with QS, i.e. proteins that were differentially expressed in mutants in the absence of signaling AHLs but were restored in the wild type by addition of external AHL to the media [56]. The study revealed 20 novel proteins whose expression was regulated by QS. Differentially expressed proteins included PrpL, a serine protease, confirmed to be regulated through QS and PhuR and HasAp that are key independent factors essential in the regulation of iron intake [56]. The results are consistent with recent studies, which have demonstrated that CF strains display significantly specialized iron metabolism capabilities [52]. Why this is the case needs further clarification since recent studies show elevated levels of various metals (including iron) in the sputum of CF patients, which correlate with disease severity [57].

Biofilm formation is one of the major traits controlled by QS in *P. aeruginosa*. Park and colleagues recently compared *P. aeruginosa* PAO1 grown under planktonic (floating as single cells) and biofilm-forming conditions [58]. Proteomic investigations performed at three time points yielded 1884 proteins. Planktonic growth showed proteins involved in DNA stability and virulence, whereas biofilm samples contained an uncharacterized protein with sequence similarity to a *P. aeruginosa* adhesion protein with self-association characteristics (AidA). This study highlights that much novel information can be gained by proteomic analysis of biofilms and these efforts could be valuable for the detection of novel drug targets [58].

In conclusion, *P. aeruginosa* invariably is one of the most studied pathogens in relation to the pathophysiology of CF. However, there are several hypermutable variants evolving rapidly due to genetic adaptation to host conditions, and these should be considered as priority for in vitro and in vivo proteomic investigations. Detailed characterization of new variants of CF isolates of *P. aeruginosa* with respect to pathogenesis and inflammation might help in resolving the growing problem of drug resistance by proposing new drug targets.

3.1.2 BCC

Burkholderia spp. are *a* ubiquitous and diverse group of Gram-negative bacteria [59]. From approximately 70 species of *Burkholderia*, 17 are capable of causing respiratory tract infections in CF patients and are termed as the BCC [34]. The most prevalent members of BCC detected in CF patients are *B. cenocepacia*, *B. multivorans*, *B. vietnamiensis*, *B. dolosa*, and *B. cepacia*. Like *P. aeruginosa*, BCCs are often associated with chronic CF infections in older patients and are challenging to treat due to their adaptive properties such as biofilm formation, hypermutability, and formation of colony variants. Being a complex set of Gram-negative bacteria, BCC shares several cellular characteristics with *P. aeruginosa*, such as virulence, adaptation, and QS. For instance, BCC employs

AHL-dependent QS to regulate virulence, biofilm formation, and population control. Genomes of about 35 strains of BCC have been sequenced and annotated, with the largest genome containing over 7000 genes (http://www.burkholderia.com/). In the past two decades, there have been several studies investigating the BCC proteome aimed at generating proteome catalogues, understanding the changes conferred through drug resistance, colony variation, and biofilm formation [60].

In order to understand the molecular differences between environmental and pathogenic strains of BCC, Park et al. compared the highly virulent *B. cenocepacia* strain isolated from a CF patient with an environmental soil isolate [61]. Ninety-six protein spots were found to be differentially expressed and 26 proteins were further identified using MS including proteins involved in virulence, QS, translation, and drug resistance [61]. Among five upregulated proteins in the clinical strain, flagellin and flagellar L-ring protein are known to be directly linked with bacterial transmissibility. A recent proteomic study involved different clones of *B. cenocepacia*, isolated 3.5 years apart and prior to death of a CF patient. The 2DE profiling detected 52 differentially expressed proteins between late (high virulent) and previously (less virulent) collected specimens. These proteins were involved in metabolic reprogramming, nucleotide synthesis, translation and protein folding, cell envelope biogenesis, and iron homeostasis. This study provides an understanding of dynamic changes undertaken by BCC in the changing host environment [62].

3.1.2.1 Secretome analysis

Secreted bacterial and cell surface proteins are essential in mediating infection and inflammation. The binding of these proteins to host cells triggers an inflammatory response that releases host cytokines such as tumor necrosis factor α and various ILs, among others to combat the infection. Proteome profiling of the cell surface of *B. pseudomallei* revealed 35 proteins, 12 of which were found to be immunogenic through human sera antibody screening [63]. A more extensive comparative LC-MS/MS characterization of outer membranes of aerosol-infective human pathogens *B. pseudomallei* and *B. mallei* revealed porins, secretins, efflux pumps, components of type VI secretion system, metal transport receptors, polysaccharide exporters, and several outer membrane proteins with currently unknown functions [64]. The type VI secretion system in *Burkholderia* has been linked to virulence of the pathogen and is known to be instrumental in causing cellular damage to the host cells [65]. Two independent 2DE analyses of secreted proteins of *B. pseudomallei* and *B. cepacia* revealed 54 and 32 potential immunogenic proteins, respectively. The secretome analysis revealed metabolic enzymes, transcription/translation regulators, potential virulence factors, chaperones, transport regulators, motility-related proteins, and hypothetical proteins [66, 67]. These studies provided one of the first 2DE proteome profiles of BCC associated with CF, revealing some key virulence proteins.

BCC secretome profiles may vary with respect to growth phase. Proteomic analysis of *B. cepacia* culture supernatants at mid-logarithmic and early-stationary phases revealed 43 proteins commonly released in both growth stages with an additional 40 proteins unique to early stationary phase. The additional proteins uniquely found at the early growth phase consisted of several virulence-associated proteins including flagellar hook associated domain protein (FliD), flagellar hook associated protein (FlgK), and TonB-dependent siderophore (Fiu) [67]. *Burkholderia pseudomallei* uses an array of diverse cell secretion systems that are poorly understood in relation to function and role in virulence. Using LC-MS/MS, Burt-nick and colleagues investigated substrates of the BCC type II secretion system (T2SS) by comparing proteomes of BCC mutants that lack the capacity of membrane transport due to mutation in outer membrane secretin and a cytoplasmic ATPase with the wild-type strain [68]. The study revealed 48 differentially expressed proteins mainly comprising of hydrolases including proteases, phospholipases, and chitinases. They also showed the link between TssM, a secreted deubiquitinase protein and T2SS. TssM is known to downregulate the host immune response by deregulating activation of NF-κB, thereby reducing host pro-inflammatory response. This study is a clear illustration of the role of the T2SS in mediating virulence by sabotaging the host defense system [68].

3.1.2.2 Effects of mucoidy on virulence

Unlike *P. aeruginosa*, BCC does not convert to a mucoid phenotype in chronic colonizations but rather the opposite as shown by Zlosnik and colleagues [69]. This same research team investigated effects of mucoidy on virulence of *B. cenocepacia* at the transcriptome and proteome levels. The iTRAQ proteomics study quantitated over 500 proteins and concluded that the nonmucoid isolates that were found in an advanced chronic stage of infection in CF had upregulated AidA (virulence factor) and AhpC (confers resistance against oxidative stress) and these proteins provided additional resistance against phagocytosis mediated by host immune system cells [70]. This study demonstrates the contribution of a nonmucoid phenotype in conferring resistance to a hostile host microenvironment. However, further studies are required to decipher the mechanism and role of mucoid conversion and its relation to pathogenic virulence.

3.1.2.3 Mechanism of drug resistance

Drug efflux pumps are well characterized in connection to drug resistance properties of multidrug-resistant bacterial pathogens [71]. BCC are known to possess several drug efflux pumps. Membrane proteome analysis of a multidrug-resistant *B. thailandensis* produced by exposure to chloramphenicol revealed overexpression of resistance nodulation division efflux pumps responsible for efflux of structurally unrelated drugs including chloramphenicol, quinolones, tetracyclines, trimethoprim, and beta-lactams

[72]. This study raises the possibility that *Burkholderia* species may be highly adaptive in vivo to expel antibiotics through induction of high expression of drug efflux pumps. Effects of a broad-spectrum antibacterial agent chitosan, a derivative of chitin, on the *B. cenocepacia* membrane were demonstrated in a recent study by Ibrahim and colleagues [73]. The authors reported 15 proteins uniquely expressed under chitosan-treatment, including four TonB-dependent siderophore receptors and two efflux pumps. The study confirms the upregulation of an efflux pump as a measure to excrete the drugs from the cells and develop drug resistance. Similar observation of increased expression of efflux pumps in chloramphenicol-resistant *B. thailandensis* was previously described by Biot et al. [72]. These studies indicate a need for carefully considering the choice of antibiotics for the treatment of BCC infections in CF as is possible that administration of one antibiotic may confer resistance to an entire class or other closely related antibiotics.

3.1.2.4 Adaptation to the host environment

BCCs are known to be successful in thriving under hostile host conditions. For instance, BCC can survive within host macrophages by arresting the maturation of phagocytic vacuoles [74]. Some adaptation strategies of BCC have been investigated using proteomics. For example, Pessi and colleagues used transciptomics and proteomics to investigate adaptive capacity of the obligatory aerobic pathogen, *B. cenocepacia*, to a microoxic in vitro environment [75]. Over 2000 proteins were identified and quantified using MS and label-free spectral counting revealing increased expression of isocitrate lyases, exopolysaccharides, and several proteases [75]. Isocitrate lyase mediated production of succinate has also been shown to be key in adaptation to hypoxia in the facultative intracellular pathogen *Mycobacterium tuberculosis* [76]. The study concluded that unlike *P. aeruginosa*, *B. cenocepacia* may not survive in anaerobic conditions, however it can very well adapt to a microoxic environment, which is typically the case in lungs of CF patients.

3.1.2.5 QS

At present little is known regarding QS in *B. cenocepacia*. To investigate the cascade of proteins regulated by QS, Inhulsen and co-workers used CepIR mutants lacking AHL in the *B. cenocepacia* strain isolated from a CF patient and compared the proteome and transcriptome profiles to the wild-type strain [77]. This analysis revealed three major proteins involved in biofilm formation, which were shown to be regulated by QS, namely BclACB lectin, type1 pilus, and the large surface protein BapA. Further, BapA mutants demonstrated its essential role for the formation of biofilms on abiotic surfaces [77]. For the first time, this study revealed a network of proteins controlled by QS in *B. cenocepacia*.

To summarize, proteomic studies of BCC have uncovered a complex network of proteins involved in QS, adaptation to

nutrient and oxygen-deficient environments, and drug resistance. However, the field lacks in vitro studies that consider the behavior and properties of the pathogen in CF-mimicking conditions that are becoming available for *P. aeruginosa*.

3.2 Fungal proteomics

Compared to bacteria, there have been fewer proteomic studies on pathogenic fungi in the context of CF. This is in spite of the fact that a wide range of fungal species have been detected from the sputum expectorant of immunocompromised CF patients [78]. However, unlike bacterial infections, it remains to be resolved whether fungal airway infections are associated with severe pulmonary exacerbation. One of the most commonly detected fungi, *Aspergillus fumigatus* causes allergic bronchopulmonary aspergillosis in CF patients [34,79,80]. However, the prevalence of other fungi in CF is often underestimated due to inconsistencies in the culture techniques used to isolate them from the sputum, BALF, and other respiratory tract samples obtained from CF patients [81,82]. Application of selective culture conditions specific for fungi and incorporation of molecular techniques such as microarray hybridization and DNA fingerprinting in the current diagnostic practices have facilitated the identification of more diverse fungal species such as *Scedosporium* spp., *Exophilala dermatidis*, and *Acrophialophora fusispora* [78,83,84]. Among them, *Scedosporium* spp. is the second most common filamentous fungus associated with CF in Australia [83]. Apart from filamentous fungi, certain yeasts such as *C. albicans* are also known to cause a decline in CF lung function, which requires treatment with antibiotics, glucocorticosteroids, and probiotics [85,86]. It is obvious that the current treatment strategies for fungal infections in CF are limited owing to the high resistance of most fungal species to the currently available antifungal agents [87,88]. Therefore, greater understanding of the clinical relevance of fungi in CF pathogenesis is required to enhance the treatment of CF patients.

3.2.1 *Aspergillus* spp

Aspergilli are saprophytic filamentous fungi found in the environment and represent fungal species most commonly associated with CF. *Aspergillus* species such as *A. fumigatus* are known to adapt to the harsh environments of the lungs where they are exposed to oxidative stress, hypoxia, and limited nutrients. Research using proteomics as a tool has been carried out to understand the cellular mechanisms underpinning these adaptations [80]. Most of the proteomic studies on filamentous fungi in the context of CF have been conducted on *A. fumigatus*.

In a given environment, oxygen availability can be categorized into three classes: normoxic (atmospheric levels of generally 21% O_2), hypoxic (reduced availability of oxygen compared to atmospheric levels), and anaerobic or anoxic

(complete absence of oxygen) [89]. Hypoxia is one of the major challenges fungi face in the CF lung environment to which they adapt and continue to infect the cells [89]. To understand the mechanism of adaptation of *A. fumigatus* to hypoxic conditions in vitro, Barker and colleagues cultivated the fungus in an oxygen-controlled fermenter under normoxic (21% O_2) and hypoxic (0.2% O_2) conditions for up to 24 h and compared proteomic and transcriptomic profiles [90]. The authors observed decreased expression of the tricarboxylic acid (TCA) cycle and oxidative phosphorylation enzymes suggesting a major metabolic switch to overcome stress. Simultaneously, there was an increase in proteins related to glycolysis, fermentation, response to oxidative stress, cell wall biosynthesis, and iron metabolism [90]. Additionally, upregulation of proteins involved in ergosterol biosynthesis and the γ-aminobutyric acid (GABA) shunt were observed. Ergosterol is required for the formation of cell membranes, and proteins involved in the GABA shunt are essential for bypassing the TCA cycle in hypoxic conditions and they are also a common fungal drug target. The authors postulated that increase in iron uptake was linked to the increase in ergosterol biosynthesis enzymes that require iron as a cofactor. These studies suggest that the ergosterol biosynthesis pathway is key in helping the fungal cells to cope with the cellular stresses induced by hypoxia. Compared to the above study [90] in which the fungus was exposed to hypoxia only for a short period, Vodisch and colleagues investigated the effects of longer exposure to hypoxia and concluded that oxidative respiration is important in long-term hypoxia, which is typically the condition in CF lungs and fermentation might only be related to short-term hypoxia [91]. Overall, these studies suggest that in CF lungs, *A. fumigatus* deviates from the normal metabolic pathways such as TCA cycle to avoid self-damage through reactive oxygen species produced during TCA cycle and manages the scarcely available oxygen by switching into alternative pathways such as the GABA shunt.

While CF patients can be prescribed with antifungal medication, resistance to these drugs is an evergrowing threat [78,92]. One example compound is caspofungin, a lipopeptide that blocks the enzyme (1→3)-β-D-glucan synthase, inhibiting the synthesis of the main fungal cell wall component, (1→3)-β-D-glucan. To investigate the cellular fine tuning that occurs in response to this antifungal compound, Cagas et al. examined a caspofungin-susceptible and -resistant strain of *A. fumigatus*. Cytoplasmic, cell wall, secreted, and microsomal proteins were analyzed using 2DE and MS coupled with iTRAQ quantification [93]. A total of 122 proteins displayed at least a twofold change in expression between the treated strains including chitinase ChiA1 (downregulated) and AspF3, a thioredoxin peroxidase (upregulated). Enolase that is a glycolytic enzyme and strong stimulator of human gamma interferon was also upregulated. Hence, this study revealed some key proteins involved in critical stress responses including antioxidants such as thioredoxin peroxidase, which have roles in fungal drug resistance [93]. The activity of the wide-spectrum antifungal agent amphotericin B against

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A. fumigatus has been studied by proteomics. *Aspergillus fumigatus* was treated with amphotericin B, which led to the downregulation of proteins involved in the formation and maintenance of cell wall; chitinase, cell wall tir-3 protein, and a conidial cell wall protein hydrophobin RodBp. The upregulated proteins represented chaperones and other stress response pathway proteins including Mn-superoxide dismutase and glutathione *S*-transferase [94]. There have been several other proteomic studies on pathogenic fungi in a non-CF context that have been extensively reviewed elsewhere [95].

Unlike bacterial pathogens, fungi are eukaryotes and thus possess cellular mechanisms similar to that of the host (humans), which makes targeted drug development and treatment much more challenging. The above studies provide a window into proteins that are differentially expressed by fungi as part of a mechanism for adaptation to the CF lung environment.

4   Future directions

Pathophysiology of CF cannot be merely explained by mutation in a single gene. Other factors such as infection and inflammation clearly exacerbate patient symptoms. In the past decade, proteomics has served as a useful tool for improved understanding of the nature and biological effects of the CFTR mutation, inflammation processes, and microbial infection. In addition, these studies have contributed toward understanding of microbial virulence and drug resistance. In summary, this field has progressed from being merely a technique for cataloguing proteins encoded by a genome, to an approach that can be used to quantify and profile changes in the levels of proteomes and their PTMs in the context of CF. In the coming years, proteomics promises to contribute to greater understanding of CF biology by supporting the analysis of more complex experiments. Areas of expected growth include: (i) robust and standardized proteogenomic workflows enabling proteomics to take advantage of the genome sequencing data of clinical microbial isolates, and (ii) more sophisticated in vitro experiments representing CF-associated infections that are complex in nature involving multiple pathogens, usually in mixed biofilms. For proteomics, this means extracting information from mixed species or pathogenic communities. The field has only begun to explore computational solutions to these types of questions [96]. Importantly, since many of the proteomic studies associated with pathogens have utilized laboratory strains, it will be increasingly necessary to characterize clinically isolated strains from CF patients, to explore the moving target of complex infections and the evolution of multidrug resistance.

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Chapter-3

Genetically and phenotypically distinct *Pseudomonas aeruginosa* cystic fibrosis isolates share a core proteomic signature

Rationale:

This chapter contains a published original research article;

Genetically and phenotypically distinct *Pseudomonas aeruginosa* cystic fibrosis isolates share a common proteomic signature. Anahit Penesyan, Sheemal S Kumar, <u>Karthik Kamath</u>, Abdulrahman M Shathili,Vignesh Venkatakrishnan, Christoph Krisp, Nicolle H. Packer, Mark P. Molloy, Ian T. Paulsen. *PLoS One*. 2015 Oct 2;10(10):e0138527.

This chapter describes an integrative genomic, proteomic and phenotypic characterization of four novel strains of *P. aeruginosa* (PASS1-4) isolated from sputum of cystic fibrosis patients. Comparison of results between PASS strains with non-CF standard laboratory strain PAO1 enabled to gain insights into diversities in colonization and virulence-related traits of CF isolates that may provide a survival advantage to the bacterium in CF lungs.

Contribution:

Isolation of the *P. aeruginosa* strains from CF sputum was performed by Dr. Vignesh Venkatakrishnan. Genome sequencing phenotypic assays, data interpretation was performed by Dr. Anahit Penesyan. Bacterial cell culture for proteomic experiments and method optimization for protein extraction and mass spectrometry sample preparation was performed by me and Sheemal Kumar (Equal contribution). Mass spectrometry (MS) analysis, MS data processing, statistical analysis and data interpretation, was performed by me and Dr. Christoph Krisp (Equal contribution). Proteomics and mass spectrometry methods were written by me.

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RESEARCH ARTICLE

Genetically and Phenotypically Distinct *Pseudomonas aeruginosa* Cystic Fibrosis Isolates Share a Core Proteomic Signature

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Competing Interests: The authors have declared that no competing interests exist.

Abstract

The opportunistic pathogen *Pseudomonas aeruginosa* is among the main colonizers of the lungs of cystic fibrosis (CF) patients. We have isolated and sequenced several *P. aeruginosa* isolates from the sputum of CF patients and compared them with each other and with the model strain PAO1. Phenotypic analysis of CF isolates showed significant variability in colonization and virulence-related traits suggesting different strategies for adaptation to the CF lung. Genomic analysis indicated these strains shared a large set of core genes with the standard laboratory strain PAO1, and identified the genetic basis for some of the observed phenotypic differences. Proteomics revealed that in a conventional laboratory medium PAO1 expressed 827 proteins that were absent in the CF isolates while the CF isolates shared a distinctive signature set of 703 proteins not detected in PAO1. PAO1 expressed many transporters for the uptake of organic nutrients and relatively few biosynthetic pathways. Conversely, the CF isolates expressed a narrower range of transporters and a broader set of metabolic pathways for the biosynthesis of amino acids, carbohydrates, nucleotides and polyamines. The proteomic data suggests that in a common laboratory medium PAO1 may transport a diverse set of "ready-made" nutrients from the rich medium, whereas the CF isolates may only utilize a limited number of nutrients from the medium relying mainly on their own metabolism for synthesis of essential nutrients. These variations indicate significant differences between the metabolism and physiology of *P. aeruginosa* CF isolates and PAO1 that cannot be detected at the genome level alone. The widening gap between the increasing genomic data and the lack of phenotypic data means that researchers are increasingly reliant on extrapolating from genomic comparisons using experimentally characterized model organisms such as PAO1. While comparative genomics can provide valuable information, our data suggests that such extrapolations may be fraught with peril.

Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic disorder affecting most critically the lungs, and also the pancreas, liver, and intestine. It is characterized by abnormal transport of chloride and sodium ions across the epithelium leading to thick, viscous secretions. It is most common among the Caucasian population where, according to the World Health Organisation, approximately 1 in 2500 children are born with the genetic mutation leading to the development of CF. CF is caused by mutations in the gene encoding for the CF transmembrane conductance regulator protein (CFTR) involved in the regulation of the movement of chloride and sodium ions across epithelial membranes. The thick mucus that forms as a result of *CFTR* mutations represents a breeding ground for various microorganisms that cause chronic infection in lungs, thus leading to complications associated with the disease.

Pseudomonas aeruginosa, a Gram-negative opportunistic human pathogen, is one of the main colonizers of lungs in CF patients. It was found that by the age of 3 years, over 95% of children with CF show evidence of intermittent *P. aeruginosa* infection [1]. Moreover, early colonization with *P. aeruginosa* has been strongly correlated with poor prognosis in CF [2, 3]. By the age of 25, according to the 2010 Cystic Fibrosis Foundation Patient registry Annual Data Report, *P. aeruginosa* becomes the most dominant microorganism in the respiratory tract of CF patients.

Due to its metabolic versatility, innate resistance to the majority of drugs used in clinical practice, and extensive biofilm formation, infections caused by *P. aeruginosa* are especially hard to treat using conventional treatment regimes and are often destined to fail. As a result, CF is ranked among the most widespread life-shortening genetic diseases with the current life expectancy often not exceeding mid 40s.

Successful CF pathogens, including *P. aeruginosa*, have developed an effective arsenal to establish infection and evade the host response, together with an ability to adapt readily to the lung environment [4]. Thus, according to reports, isolates of *P. aeruginosa* that are involved in the acute infection and initial colonisation of CF lungs in early childhood differ from those found in adults with established chronic infections, the latter often showing adaptations specific to the CF lung environment [5, 6].

Considering its role in the disease progression, it is not surprising that *P. aeruginosa* has become a focal point for research in CF and other biofilm related complications. Improvements in DNA sequencing technology have led to the sequencing of hundreds of *P. aeruginosa* genomes in recent years, many of them from CF patients. However, for the vast majority of these strains there is little or no published experimental data on their phenotypic features ([Fig 1]). Instead most phenotypic experimental work on *P. aeruginosa* has focused on common well-characterised model strains, such as PAO1. Our knowledge of these sequenced CF isolates is thus largely based on extrapolations from model strains via *in silico* genome comparisons.

Strain PAO1 is a widely used model organism and the first *Pseudomonas* species to have its genome sequenced [7]. In 2014 alone 155 articles were published, as listed on NCBI PubMed, using PAO1 as a model *P. aeruginosa* strain, and 15% of those articles made direct correlations to CF. As strain PAO1 does not have a CF origin but was isolated from a wound infection in 1955 [8] and has been maintained in laboratories worldwide since then, PAO1 may have questionable relevance to CF.

In this study we have obtained fresh isolates of *P. aeruginosa* from the sputum of CF patients, subsequently named PASS1-4, and compared them with each other and with the model laboratory strain PAO1. The study aimed to identify specific adaptations developed by *P. aeruginosa* during chronic infection of CF lungs with an overarching aim of better understanding the mechanisms that render *P. aeruginosa* a successful CF lung colonizer. Significant

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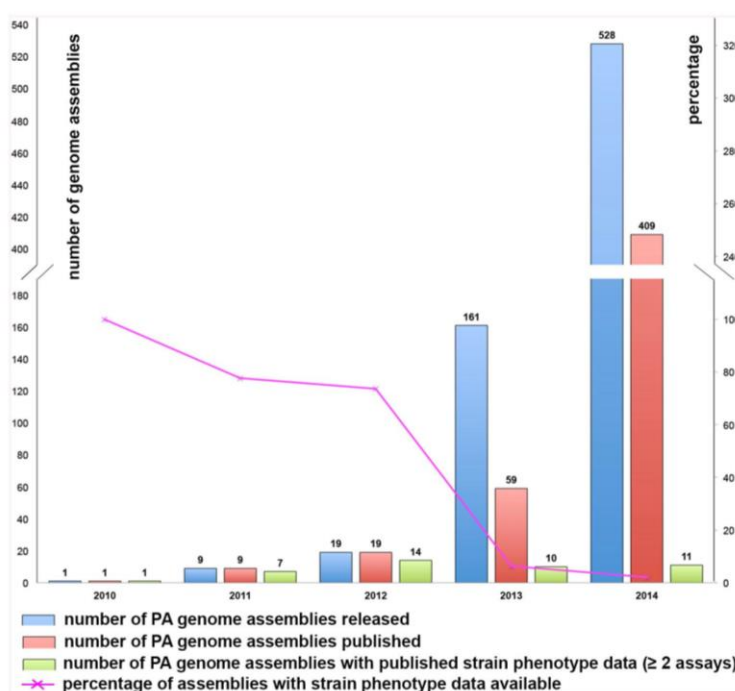
Fig 1. Data on *P. aeruginosa* (PA) genome assemblies released in the last 5 years (2010–2014 inclusive).

differences were observed in genomes and phenomes amongst the CF isolates reflecting varying adaptation paths. At the same time, the CF isolates shared a common proteomic signature that was remarkably distinct from the proteome of PAO1.

Our study highlights the limitations of using model organisms when examining the role of bacteria in the context of their natural host/environment. Isolates from hosts/environments of interest may have developed specific adaptations that may not be present in model strains, or may have been lost during prolonged cultivation. Understanding these adaptations would be crucial for the successful treatment of infections caused by pathogens; therefore, the use of freshly obtained isolates is an important adjunct to work on model strains.

Materials and Methods

Strains used in this study

P. aeruginosa isolates PASS1-4 were previously obtained from the sputum of adult CF patients: PASS1 was obtained from a 40-year old female patient, PASS2 –from a 27-year old male, PASS3 –from a 23 year old male and PASS4 –from a 23-year old female [9]. Strains PASS1-4, as well as *P. aeruginosa* PAO1, were maintained in stock in -80°C freezer and grown on Luria Bertani (LB) solid or liquid media whenever needed unless otherwise stated, allowing minimal passaging before the assays.

Genome sequencing, assembly and comparative genomics

DNA was isolated from *P. aeruginosa* strains using the Invitrogen PureLink Genomic DNA kit and sequenced at the Ramaciotti Centre for Gene Function Analysis (UNSW, Sydney) on an Illumina HiSeq 2000 platform. Sequence data was assessed for quality using FastQC (Babraham Bioinformatics) and assembled *de novo* using the VELVET algorithm [10]. Genome assembly quality and statistics were further evaluated using QUAST [11].

Genome sequencing data for strains PASS1-4 have been deposited to the NCBI Whole Genome Shotgun (WGS) database and are accessible under BioProject ID numbers PRJNA295120, PRJNA295121, PRJNA295122 and PRJNA295123 respectively (http://www.ncbi.nlm.nih.gov/bioproject).

The phylogenetic relationship of PASS1-4 strains to other *P. aeruginosa* strains in the MLST database (http://pubmlst.org/paeruginosa) was inferred via maximum likelihood analysis using the Arb software package [12]. The phylogenetic analysis was based on concatenated nucleotide sequences of seven genes utilized in the multilocus sequence typing (MLST) of *P. aeruginosa*. These included *acsA* (acetyl coenzyme A synthetase), *aroE* (shikimate dehydrogenase), *guaA* (GMP synthase), *mutL* (DNA mismatch repair protein), *nuoD* (NADH dehydrogenase I chain C, D), *ppsA* (phosphoenolpyruvate synthase), *trpE* (anthralite synthetase component I). Further analysis has been performed by assigning a number to each distinct allele within a locus according to the number available in the *P. aeruginosa* MLST database. As a result, each isolate was given up to seven numbers that represented its strain type. Any strain type that did not have a match in the existing database was designated as a "new" type.

Multiple genome alignments were performed using BRIG [13] and MAUVE [14] software.

Reciprocal similarity searches of all predicted and expressed proteins were performed using BLAST algorithm [15], as well as Proteinortho5 tool [16] followed by the visualization of results using FriPan (http://www.vicbioinformatics.com/software.fripan.shtml).

Carbon source utilization

Carbon source utilization was assessed by growing bacteria in M9 minimal medium (Sigma) with the addition of the substrate of interest as a sole carbon source, in biological triplicates. Cultures were incubated on a horizontal shaker for 48 hours at 37°C after which the growth was assessed.

Biofilm formation in flow cells

To assess formation of biofilms by *P. aeruginosa*, strains PASS1-4 and PAO1 were grown in continuous flow-cell system [17] in LB medium at 37°C for 48 hours and biofilms formed on the surface of coverslips were imaged using Olympus FV1000 Laser Scanning Confocal Microscopy (LSCM) System after staining with the BacLight Live/Dead stain (Molecular Probes). Three-dimensional rendering of LSCM images and the subsequent quantification were performed using the Imaris software (Bitplane). Quadruplicate images were used in the quantitative assessment of LSCM images of each sample; these included two independent flow cell chambers that were inoculated from separate culture stocks, and two distant fields of view in each chamber.

Binding of *Pseudomonas aeruginosa* to mucin

P. aeruginosa isolates were cultured in 10 ml of LB broth at 37°C for 8 hours with shaking at 185 rpm. Bacterial cells were pelleted by centrifugation at 3000 x *g* for 5 min, washed twice with phosphate buffered saline (PBS), and then fluorescently labelled by resuspension in 1 ml

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of PBS with SYBR® Green (0.1% w/v, Sigma) for 3 min. Labelled cells were collected by centrifugation at 3000 x g for 3 mins, and then washed thrice in PBS to remove residual dye. Meanwhile, PVDF membranes were placed into the wells of a 96-well microtiter plate and activated by soaking in methanol followed by washing three times in PBS. Fifty microliters of 1 mg/ml porcine gastric mucin (PGM, Sigma) were added into each well containing a PVDF membrane. Bacteria were resuspended in PBS to an $OD_{600} = 1.0$, applied to the wells of 96-well microplate containing the mucin-coated PVDF membranes and incubated for 30 minutes while slowly shaking at 100 rpm at RT. Unbound bacteria were washed off the membrane three times with PBS and the attached bacteria were fluorescently measured (Ex 485nm, Em 520nm) using a Fluorostar Galaxy plate reader (BMG Labtech, Offenburg, Germany). Wells containing immobilised mucin (no bacteria applied) were used as a negative control. The binding to the mucin by the different bacterial strains was normalized against the maximum bacterial binding measured on each plate.

Flagella mediated motility

Flagella mediated motility was assayed by point inoculation of LB plates containing 0.3% (w/v) agar as previously described [18]; zone sizes were observed after 48 hours of incubation at 37°C. The assay was performed in biological triplicates.

Virulence against the nematode eukaryotic model *Caenorhabditis elegans*

To assess toxicity against the nematode *C. elegans*, a selective grazing assay was performed as previously described [19]. Briefly, all strains of *P. aeruginosa* were spotted from frozen stock cultures on a single LB agar plate, followed by incubation at 37°C for 4 days. At day 4, 5 μl of M9 medium with four to five L4-stage nematodes were added next to each colony. The plates were stored at room temperature and checked daily. A strain was considered positive if the colony was not grazed after 14 days of incubation with *C. elegans*, across all three biological replicates. The assay was performed in three biological replicates.

Assessment of phenazine production

Phenazine compounds were extracted from *P. aeruginosa* cultures (in biological triplicates) after 40 hours of growth in LB broth, at 37°C with shaking. Two millilitres of chloroform were added to 5 ml of culture and incubated with shaking for 20 minutes. Chloroform fractions were collected, dried under reduced pressure and dissolved in 80% v/v acetonitrile in 25 mM ammonium acetate. Filtered samples were applied to the Zorbax Eclipse Plus C18 Rapid Resolution column (Agilent, 2.1 x 50 mm, 1.8 micron) and analysed using the Infinity 1290 Ultra High Performace Liquid Chromatography (UHPLC) instrument equipped with the Infinity 1290 photodiode array detector (Agilent). The separation program was adopted for UHPLC based on the previously published protocol [20]. The solvent flow rate was 0.5 ml/min and consisted of 0.25 minutes of 8% v/v acetonitrile–25 mM ammonium acetate, followed by a 3-min linear gradient to 80% v/v acetonitrile–25 mM ammonium acetate. UHPLC gradient profiles were monitored at spectral peak maxima of 257.0 and 313.0 nm.

Pyoverdine production

Pyoverdine production was assessed by growing strains on iron-limited King's medium at 37°C for 40 hours, in biological triplicates, followed by visualization of pyoverdine under UV light, as previously described [21].

Protein extraction for proteomics

Overnight cultures of *P. aeruginosa* strains (PAO1, PASS1, PASS2, PASS3 and PASS4) in LB broth, in biological triplicates, were inoculated into fresh LB broth and grown to mid-logarithmic phase with incubation at 37°C and shaking at 200 rpm. Cells were collected by centrifugation at 2500 x *g* for 10 min at 4°C and washed thrice with PBS, pH 7.4. The cell pellet (~ 0.5g) was weighed and resuspended in 0.5ml of PBS (pH 7.4) containing complete protease inhibitor cocktail (EDTA-free, Roche), benzonase (1:100 v/v, Sigma) and equal amounts of acid washed glass beads (Sigma Aldrich). Cells were lysed by bead-beating thrice at 4.5 Throw for 20 seconds using a FastPrep FP120 bead-beater apparatus (Savant) with 10 min breaks on ice. Cell debris and unbroken cells were removed by centrifugation at 2500 x *g* for 8 min at 4°C. Proteins in the supernatant were precipitated by the addition of ice-cold acetone in the ratio of 1:9 (v/v) and incubated overnight at -20°C followed by centrifugation at 2500 x *g* for 10 min at 4°C. The pellet was washed twice and resuspended in 1% (w/v) SDS in deionized water. Total protein content was measured using Pierce BCA Protein Assay Kit (Thermo Scientific).

1D SDS-PAGE and in-gel digestion

Thirty micrograms of protein from each sample was diluted using NuPAGE Laemmli loading buffer (Life Technologies) containing 50 mM DTT and denatured at 95°C for 5 min. Samples were spun down and loaded into a NuPAGE (4–12% T Bis-Tris) precast ready gel. Electrophoresis was performed using NuPAGE MOPS SDS running buffer to run the sample 10 mm into the gel. After electrophoresis the gels were stained with colloidal Coomassie blue (Sigma Aldrich) and the band was cut into 1 mm³ cubes and transferred into 1.5 ml microcentrifuge tubes to perform in-gel tryptic digestion.

Trypsin in-gel digestion and peptide extraction were performed as previously described [22]. Briefly, gel bands were destained using acetonitrile (ACN) and 100 mM ammonium bicarbonate (ABC) in the 1:1 ratio, followed by reduction with 10 mM DTT prepared in 100 mM ABC at 56°C for 30 min and alkylation with 55 mM iodoacetamide prepared in 100 mM ABC for 20 min in the dark. The gel plugs were washed twice with ACN, with 5 minutes of incubation during each wash. The gel plugs were subsequently dried in a vacuum centrifuge, and digested overnight with sequencing-grade modified trypsin (Promega) in the ratio 1:30 at 37°C. Products of digestion were collected in 1.5 ml microcentrifuge tubes and combined with extracts from a consecutive extraction with ACN and 5% (v/v) formic acid at 37°C for 30 min. The total extract was then concentrated in a vacuum centrifuge and reconstituted with loading buffer (2% v/v ACN, 0.1% v/v FA) for mass spectrometry.

Mass spectrometry

Peptides were analysed using a data-independent acquisition method known as SWATH-MS [23]. Five spectral ion libraries were generated, one for each strain by pooling biological replicates and separating the peptides into five salt fractions by online strong cation exchange chromatography (SCX) These libraries were later used for proteomics data analysis. Fractionated samples were analysed using a nanoLC ultra 2D cHiPLC system (Eksigent, part of SCIEX) in conjunction with a TripleTOF® 5600 (ABSciex) using positive nanoflow electrospray analysis and an information-dependent acquisition (IDA) mode. In data dependent MS/MS acquisition 20 most intense m/z values exceeding a threshold > 150 counts per second (cps) with charge stages between 2+ and 4+ were selected for analysis following a full MS survey scan and excluded for 20 sec to minimize redundant precursor sampling.

For SWATH-MS, each biological triplicate was analyzed within a 60 min increasing ACN RP gradient (5% to 45% using 90% v/v ACN 0.1% v/v FA) using 60 variable window m/z

ranges (400–1250 m/z) selected based on intensity distribution of precursor m/z in the IDA data sets. Collision energies were calculated for 2+ precursors with m/z values of lowest m/z in window + 5 m/z and a collision energy spread of 5 eV was used.

Protein identification

Spectral libraries for SWATH-MS quantitation were generated with ProteinPilot[TM] software 4.2 using the Paragon[TM] algorithm (ABSciex) thorough ID mode including biological modifications [23]. MS/MS data were searched against the *Pseudomonas aeruginosa* strain PAO1 protein sequence database retrieved from GenBank (January 2013) and Pseudomonas Genome Database (www.pseudomonas.com) [24] and *in-silico* translated genome databases of PASS1-4 strains. Carbamidomethylation of Cys residues was selected as a fixed modification. An Unused Score cut-off was set to 2.0 (99% confidence), equivalent to a protein false discovery rate (FDR) < 1%.

Proteomic data analysis

Generated protein libraries for each strain were imported into PeakView[TM] software 2.1 using the SWATH MicroApp 2.0 (release 27 November 2013) and matched against SWATH-MS data for each individual replicate. After retention time calibration with endogenous peptides, data were processed using the following settings; a maximum of 100 peptides per protein, maximal 6 transitions per peptide, peptide confidence threshold of 60%, transition FDR < 1%, 10 min extraction window and fragment extraction tolerance of 75 ppm.

Expressed proteins were determined by comparing summed protein areas from extracted ion chromatograms between PAO1 and PASS1-4 strains to identify those with a +/- 5-fold change and ANOVA p < 0.01.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository [25] with the dataset identifier PXD002865.

In order to reveal similarities and differences in the proteomics profiles of *P. aeruginosa* strains tested, the reciprocal Blast searches and orthologue analyses were performed as was previously described for the predicted proteome.

Proteins identified as being expressed by *P. aeruginosa* strains during the growth in LB medium were mapped onto the *P. aeruginosa* PAO1 curated database in BioCyc [26] and metabolic pathways visualized using the Pathway Tools 17.5 cellular overview diagram and Omics Viewer [27].

Results and Discussion

Colony morphology of isolated CF strains

Four *P. aeruginosa* isolates were obtained from the sputum of CF patients at the Westmead Hospital (Sydney, Australia) and were minimally passaged. Isolates displayed significant diversity in their colony morphology: PASS1 formed pale-green non-mucoid colonies when grown on LB agar, PASS2 –light brown non-mucoid colonies, PASS3 –mainly white mucoid colonies and PASS4 –dark green-blue non-mucoid colonies.

Whole genome sequencing and phylogeny

De novo assembly of the sequencing reads obtained from Illumina HiSeq platform yielded draft genomes as per Table 1. Strain PASS3 was found to have the largest genome, and PASS2 genome was the smallest. Based on the MLST phylogeny, strains PASS1 and PASS3 were found to have an identical MLST sequence, while PASS2 and PASS4 were in different MLST clusters

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Table 1. Genome assembly statistics for PASS1-4 strains.

Strain	Number of contigs (> 1 kb)	N50	Genome size (Mbp)	Number of ORFs
PASS1	81	205938	6.3	5792
PASS2	96	186035	6.1	5795
PASS3	76	255936	6.4	5847
PASS4	93	171309	6.3	5936

Further MLST analysis revealed that strains PASS1-3 do not have an exact match in the MLST database (http://pubmlst.org/paeruginosa), and therefore represent new strain types, while PASS4 belongs to the strain type 649 (S1 Table). The latter currently comprises of 23 isolates one of which originates from the blood sample in Czech Republic and the rest are epidemic isolates obtained from lungs of CF patients in Australia.

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distinct from PASS1, PASS3 and PAO1 (Fig 2). Consistent with their isolation source, all CF isolates (PASS1-4) clustered with other Australian sputum isolates, while PAO1 was closely related to a more diverse set of isolates from various sources, including tissue infection strains.

Comparative genomics and the analysis of predicted proteins

Multiple genome alignments using BRIG [13] and MAUVE [14] tools show extensive gene conservation and synteny between PAO1 and the PASS1-4 genomes (Fig 3). The analysis of strain PASS2 draft genome also revealed an absence of a ~ 160 kB genomic region corresponding to the nucleotide positions between 2439150 and 2602350 in the PAO1 genome and containing genes with locus tags PA2218-PA2354 (Fig 3). This region, absent in PASS2, contains a number of potential virulence and colonization determinants, including the *psl* cluster of genes responsible for the production of biofilm matrix component Psl, as well as genes encoding chitinase and those involved in biosynthesis of L-2-amino-4-methoxy-trans-3-butenoic acid (AMB) toxin (Fig 3). This may represent an adaptation to the CF lung via genome reduction and the loss of several virulence factors. Decreased virulence has been previously described among the characteristics of *P. aeruginosa* isolates from chronic lung infections as a way of minimizing the host immune response and limiting bacterial resource expenditure [28]. A similar large deletion of ~ 180 kB spanning from PA2272 to PA2410 has been previously reported in a different CF isolate [29].

BLAST searches of predicted proteins of PASS1-4 strains and the standard laboratory strain PAO1 revealed 4676 shared protein sequences between all 5 strains (Fig 4C). Ortholog analysis of all predicted proteins performed using Proteinortho5, followed by visualization of results via FriPan demonstrated distinct clustering of PAO1 at a significant distance from CF isolates PASS1-4. Interestingly, significant differences were also observed among PASS1-4 strains, *i.e.* PASS1 and PASS3 showed high degree of similarity, while PASS2 and PASS4 clustered together with a lower level of similarity (Fig 4A and 4B), in agreement with the MLST phylogeny (Fig 2).

Carbon source utilization

Genomic analysis revealed that PASS2 lacks genes essential for the utilization of various carbon sources. Many of these genes were located in the ~160 kB region of DNA absent in strain PASS2. These include genes involved in transport and catabolism of mannitol (PA2337-PA2344); the *bkd* operon for utilization of valine, leucine and isoleucine; and genes involved in the utilization of gluconate and glycerol (PA2321-2322 and PA2352 respectively). Minimal media growth experiments confirmed that PASS2 was unable to grow in the minimal medium in the presence of the above-mentioned compounds as sole carbon sources (data not shown).
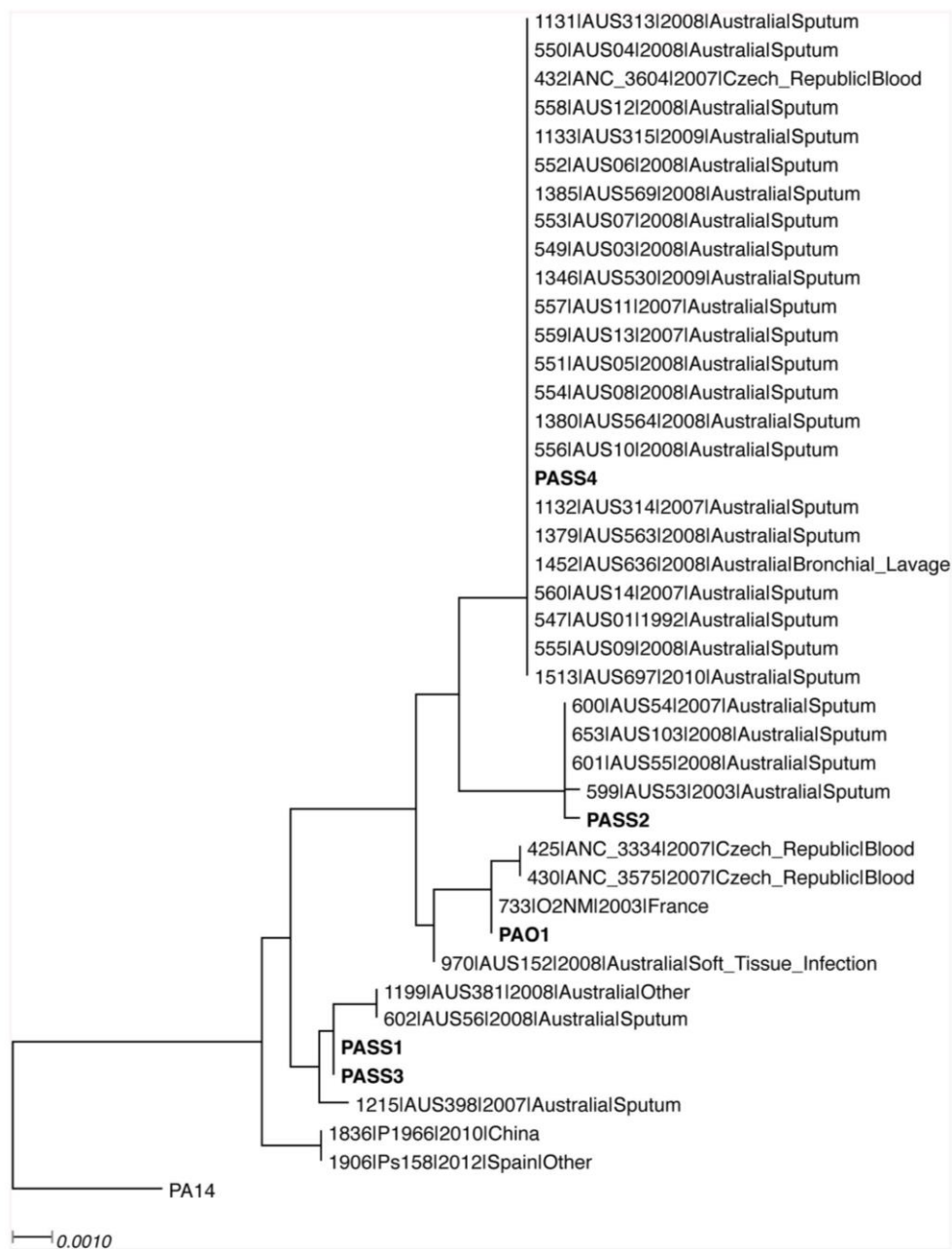
Fig 2. The maximum likelihood phylogenetic tree generated using concatenated sequences of MLST genes in *P. aeruginosa*. Isolates mentioned in this study are shown in bold; all the other closely related MLST sequences are retrieved from *P. aeruginosa* PubMLST database (http://pubmlst.org/paeruginosa). *P. aeruginosa* PA14 is used as an outgroup. The scale bar indicates the number of substitutions per nucleotide position.

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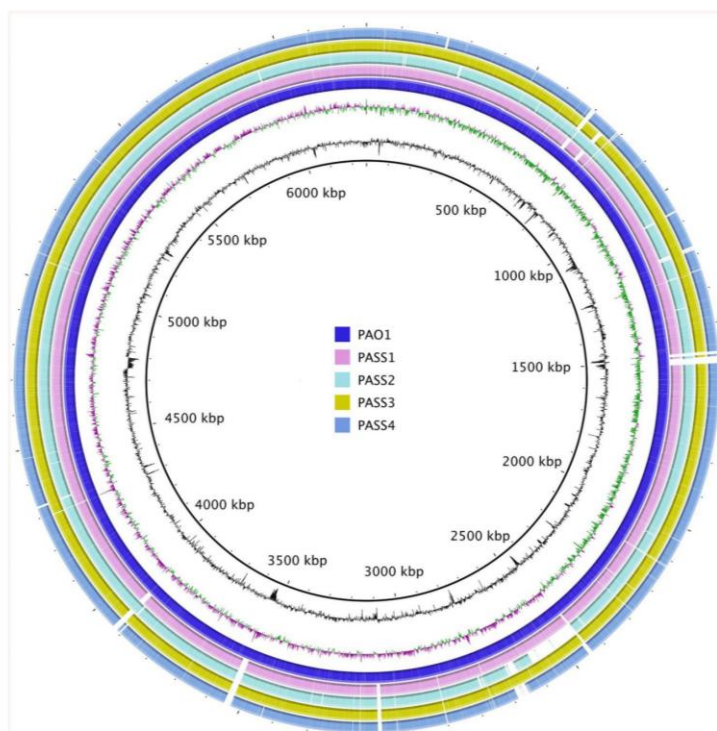
Fig 3. Circular representation of *P. aeruginosa* PASS1-4 draft genomes aligned against the reference genome of PAO1, generated using BRIG software. The two innermost circles represent the GC Content and the GC Skew respectively.

Biofilm formation

Biofilm formation in flow chambers highlighted differences in the biofilm forming ability and biofilm architecture of the *P. aeruginosa* strains. PASS1, PASS3 and PASS4 were shown to form thicker biofilms compared to PAO1 (Fig 5A and 5B). Conversely, strain PASS2 was not able to form a biofilm on the surface of glass coverslips in the flow chambers. This is likely due to the loss of the *psl* cluster of genes responsible for the biosynthesis of exopolysacharide Psl located within the PA2218-PA2354 region absent in this strain (Fig 3). Psl is one of the major exopolysaccharides involved in the formation of biofilm matrix in *P. aeruginosa*, along with alginate and Pel. Non-mucoid strains, such as PASS2, primarily utilize either the Psl or Pel polysaccharides for biofilm formation [30, 31]. Previous mutational analyses demonstrated that Psl plays an important role in surface attachment for most isolates and the Psl deficiency often leads to the lack of biofilm formation [30].

Extracellular substances like exopolysaccharide Psl, are often regarded as "public goods" as they can benefit the overall population and not only the single producer strain [32]. Production of such metabolites is often metabolically expensive. This inevitably leads to the emergence of "social cheaters"–phenotypes that do not produce certain public goods, and, therefore, do not bear the cost of their production, but still benefit from metabolites produced by other members of the community [33, 34]. Hence, it is possible that strain PASS2 may have evolved as a
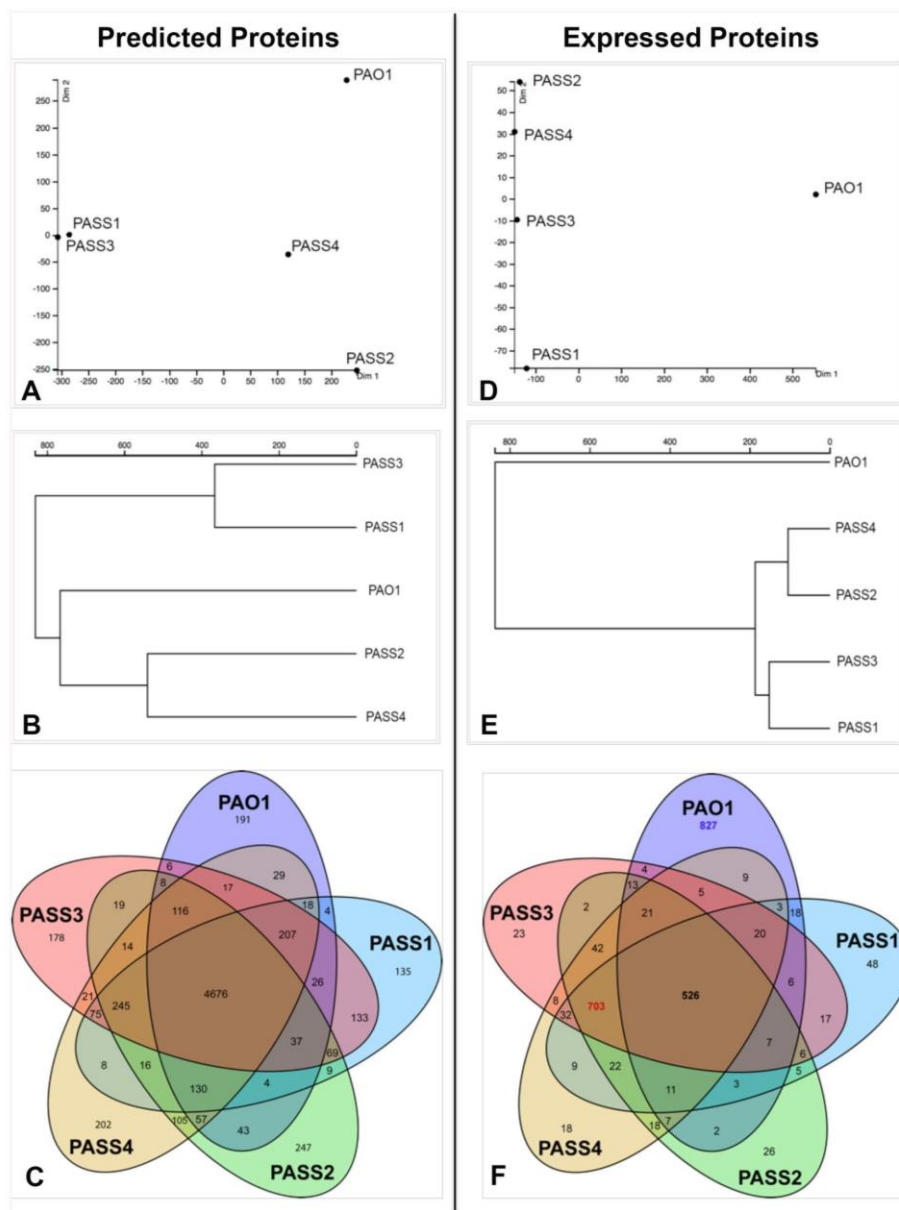
Fig 4. Comparison of predicted and expressed proteins in CF isolates PASS1-4 and the reference strain PAO1. The 2-dimensional plot (A) and clustering representation (B) of all predicted proteins of corresponding strains analysed via Proteinortho and FriPan software; (C)–Venn diagram representing the results of reciprocal BLAST searches of all predicted proteins in corresponding strains. The 2-dimensional plot (D) and clustering representation (E) of all expressed proteins of corresponding strains as assessed by proteomics and analysed via Proteinortho and FriPan software; (F)–Venn diagram representing the results of reciprocal BLAST searches of expressed proteins detected via proteomics; the number of proteins uniquely expressed by PAO1 is shown in blue, the number of proteins shared by PASS1-4 and not expressed by PAO1 is in red.

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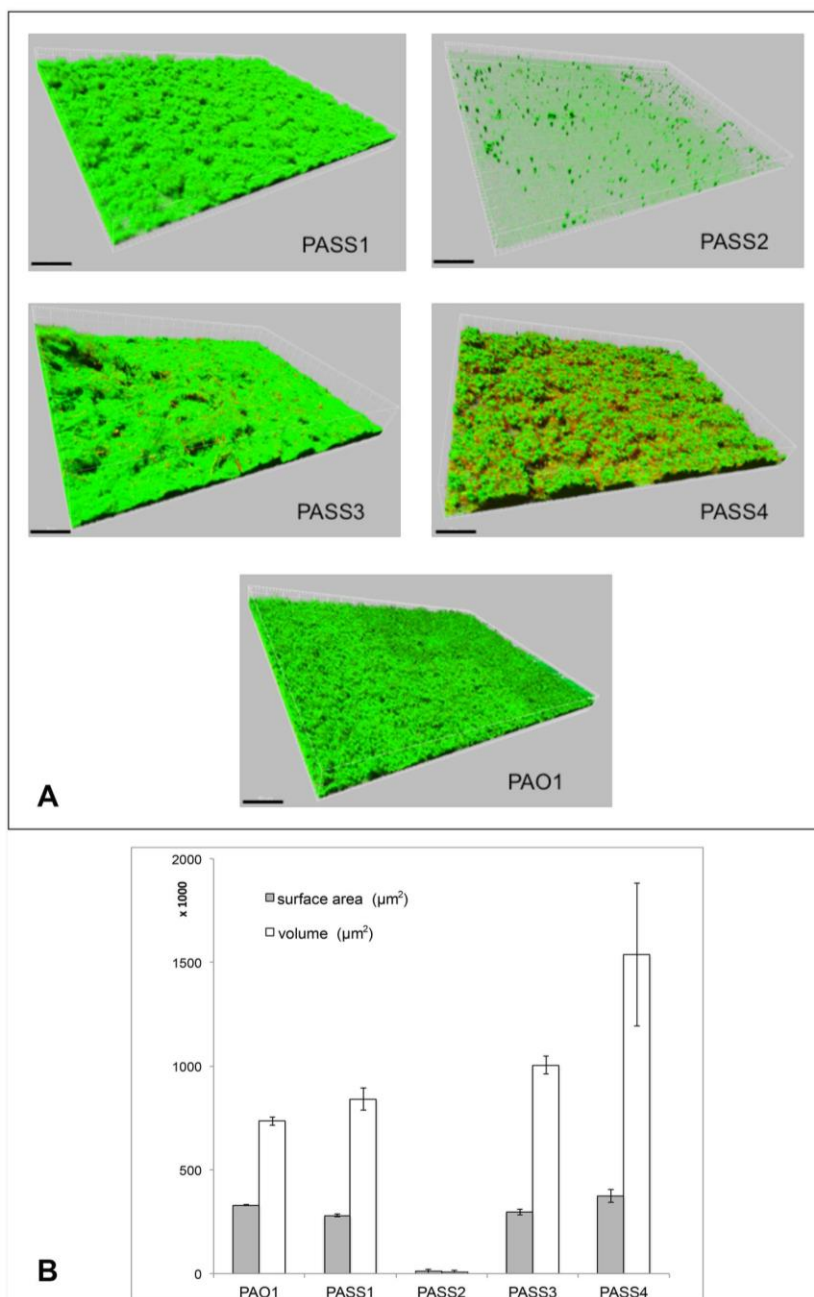
Fig 5. (A) Representative images of biofilms formed in flow cells by strains PASS1-4 and PAO1 after 48 hours of growth in LB medium at 37°C. **(B)** Quantitative analysis of quadruplicate LSCM images (derived from two independent flow cell chambers that were inoculated from separate overnight cultures, and two distant fields of view in each chamber) was performed using Imaris software. Images were taken using Olympus FV1000 confocal laser-scanning microscopy (LSCM) system, 3D pictures

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were built using Imaris software package (Bitplane). Cells are stained using BacLight Live/Dead stain (Molecular Probes); live cells are presented in green, dead cells—in red. Scale bars on LSCM images represent 50 μm.

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"cheater" phenotype and may still be able to form biofilms in the mixed communities with other strains present in the CF lung, benefiting from Psl produced by other strains. PASS2 was the only *P. aeruginosa* strain isolated from that particular CF patient, so it is not possible to directly investigate this "cheater" hypothesis.

Flagella and pili

Flagella and pili are important factors in biofilm formation, especially in the initial stages of attachment. Thus, flagella, besides being motility organelles, also play a direct role in virulence as major antigenic determinants for the immune response to *P. aeruginosa* infection [35]. PASS1, PASS3 and PAO1 shared a similar organization of flagellar biogenesis genes, while PASS2 and PASS4 showed distinct differences (S1A Fig). Based on the molecular weight and serological properties of flagellin, *P. aeruginosa* have been previously classified into two groups carrying A- and B- type flagellins [36, 37]. PASS1 and PASS3 share four genes orthologous to PA1088-PA1091 of PAO1, typical of B-type flagellins [38]. In contrast, the same region in PASS2 and PASS4 had a more complex polymorphic organisation characteristic of strains with a highly glycosylated and more heterogenous type A flagellin The duplication of the *fliS* gene observed in PASS2 and PASS4 was also characteristic for type A flagella [38].

It is largely accepted that glycosylation of flagellins is important for virulence and host specificity, however the precise physiological activity remains unclear [39, 40]. Strains PASS2 and PASS4 exhibited little or no flagellum-dependent swimming motility as opposed to PAO1, PASS1 and PASS3 (S1B Fig); whether this is a general feature of type A flagellin has yet to be elucidated.

Flagellar components have been shown to be important for adhesion to mucins [41]. Mucins are major macromolecular glycoprotein components of the mucus that line the surface of lung epithelium and that vary in their composition and bacterial adhesion properties in CF [42, 43]. Mucins are overproduced in the lungs of CF patients and binding of *P. aeruginosa* to lung epithelial mucins in CF has been previously reported [44]. The mucin-binding assay showed variations amongst the isolates, with PASS4 showing the highest level of binding to mucin. Notably, PASS2 binding to mucin was comparable to that of PASS3 (Fig 6) despite the decreased biofilm formation in flow cells (Fig 5A and 5B). This data suggests that adhesion to biological surfaces, such as to the mucins on the epithelium, is multifactorial and bacteria may employ various tools for attachment to surfaces that may allow them to at least partially compensate for a loss of another mechanism.

Bacterial type IV pili (T4P) have been extensively studied for their contribution to motility, biofilm formation and virulence [45]. T4P may, therefore, play a major role in the colonization, survival and virulence in the CF lung. Based on genomic context of T4P genes, pili are classified into groups I-V [46]. PAO1 has been shown to encode a group II pilin [46], this is also true for strains PASS1 and PASS3 based on our analyses, whereas strains PASS2 and PASS4 were found to encode group I pili. Interestingly, Kus et al. [46] have described the high prevalence of group I pili in isolates from CF patients, possibly representing a specific adaptation to the CF lung environment. Nevertheless, the significance and specific role of group I T4P in CF is currently unclear.

Phenazine production

Phenazines are secondary metabolites produced by a variety of bacteria, most notably pseudomonads, and have been studied intensively because of their broad-spectrum antibiotic
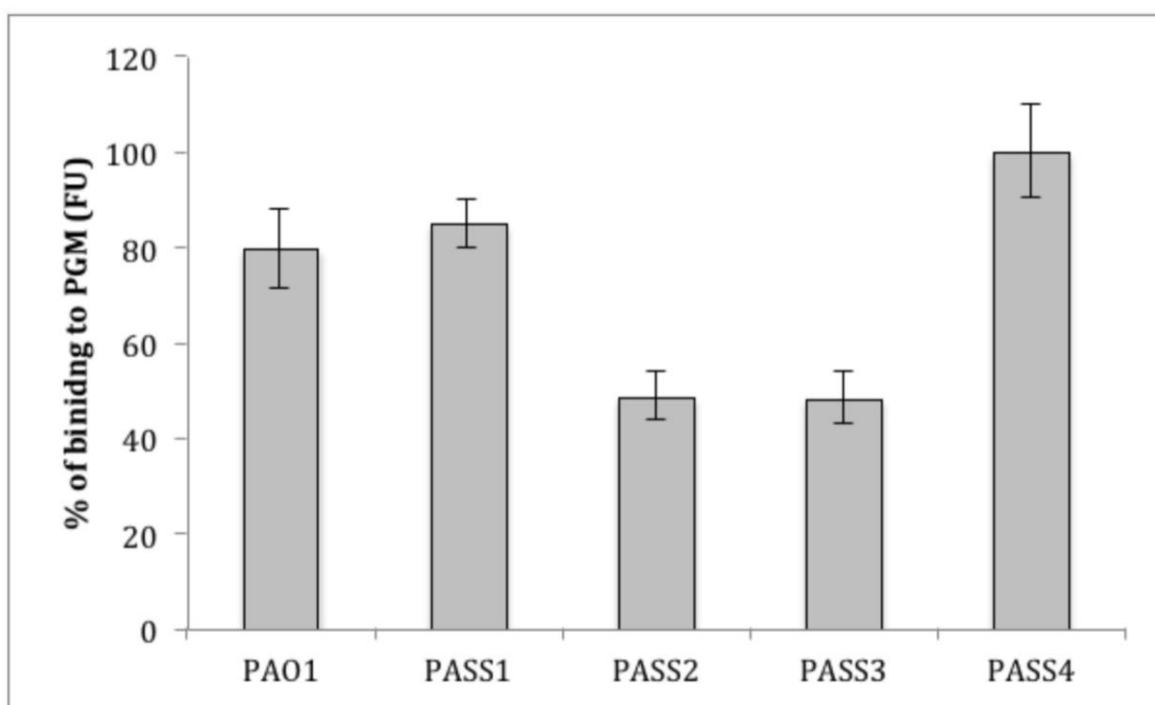
Fig 6. Binding of isolates PASS1-4 and PAO1 to porcine gastric mucine (PGM). The Y-axis represents the binding to mucin by the different bacterial strains as arbitrary fluorescent units (FU) normalised against the maximum bacterial binding of PASS4.

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properties and proven role in virulence. Many phenazine-producing bacteria are commonly found associated with host organisms [47]. The well-known phenazines produced by *P. aeruginosa* include phenazine-carboxylic acid (PCA), phenazine-carboxamide (PCN) and hydroxyl-phenazine (1-OH-PHZ), and especially pyocyanin (PYO). The latter is produced in concentrations close to 100 μmol/L during infection in CF [48, 49]; and its presence is associated with high morbidity and mortality in CF patients [50, 51].

Lau et al, using PYO-deficient mutants, have provided direct evidence that PYO is among the most potent virulence factors in the arsenal of *P. aeruginosa*. Mutants lacking PYO production were attenuated in their ability to infect mouse lungs in an acute pneumonia model of infection when compared with isogenic wild-type bacteria [52]. Ultra High Performance Liquid Chromatography (UHPLC) analyses revealed that PASS2 and PASS3 did not produce detectable phenazines (Fig 7). This was confirmed in a *Caenorhabditis elegans* selective grazing assay, where PASS2 and PASS3 were completely grazed by nematodes, indicative of a less-toxic/less-virulent phenotype, whereas all other *P. aeruginosa* colonies remained intact (Fig 8). The activity of phenazines as anti-nematode compounds against *C. elegans* has been recently demonstrated [53]. Production of the phenazine carboxamide was not observed in the strain PASS4. There is no evidence for absence or a significant disruption of genes involved in the production and modification of phenazines in any of the PASS1-4 genomes.
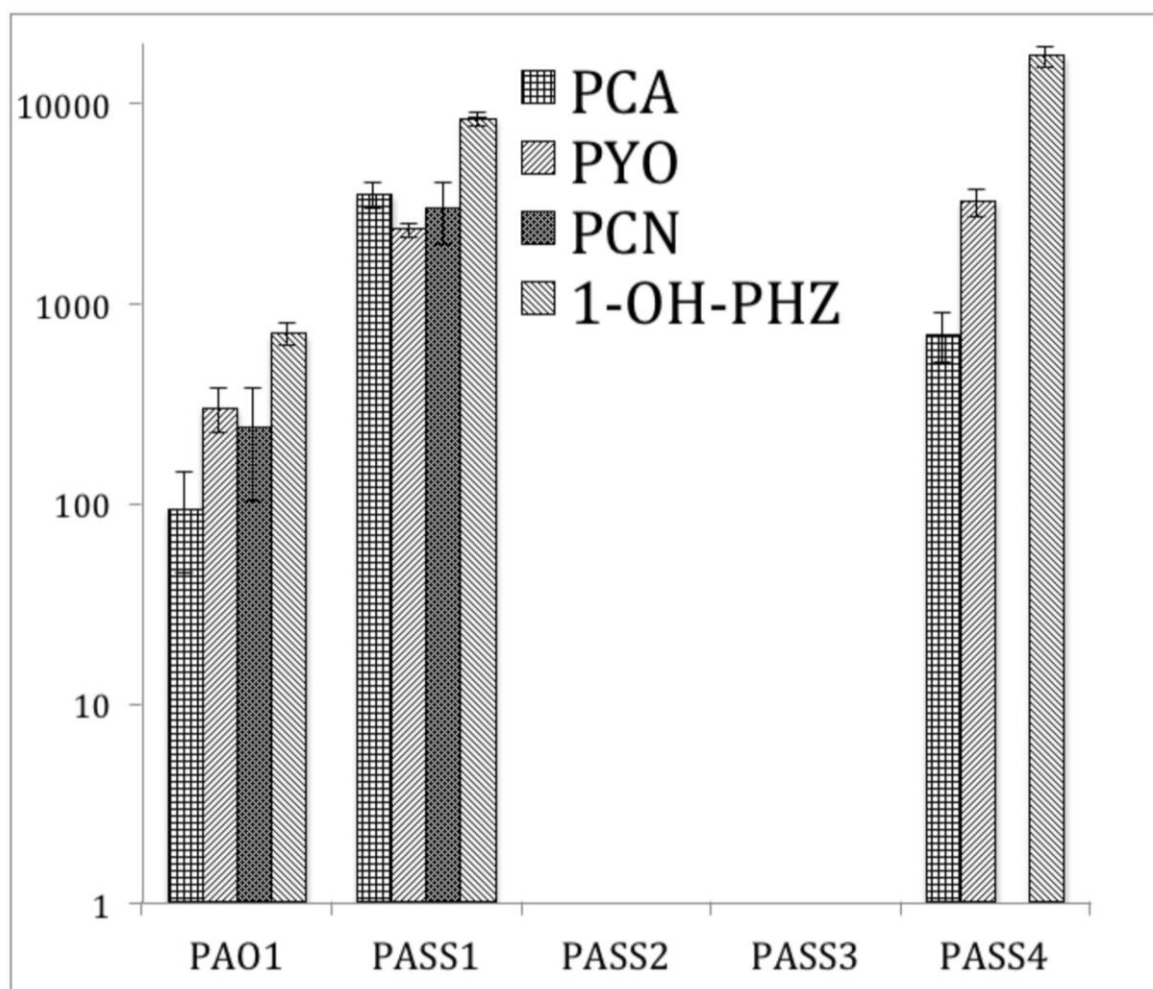
Fig 7. Production of the phenazines phenazine-carboxylic acid (PCA), pyocyanin (PYO), phenazine-carboxamide (PCN) and hydroxyl-phenazine (1-OH-PHZ) as assessed by UHPLC. The Y-axis represents arbitrary values based on chromatographic peak areas representative for each compound as observed at 257 nm, in logarithmic scale. Error bars represent standard deviations between three biological replicates.

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Production of pyoverdine

One of the main characteristics of fluorescent pseudomonads is the production a fluorescent yellow-green siderophore, pyoverdine, important for iron acquisition in low iron environments [54]. PASS2 and PASS4 did not produce pyoverdine on iron-limited King's medium, while PASS3 showed only trace amounts of pyoverdine as compared to PAO1 and PASS1 based on a pyoverdine production assay (Fig 9).

Genomic analysis revealed the loss of several genes in the pyoverdine biosynthesis pathway in both strains PASS2 and PASS4 including either complete or partial loss of genes orthologous to PA2398-PA2402 encoding the ferripyoverdine receptor FpvA, pyoverdine synthetase PvdD,
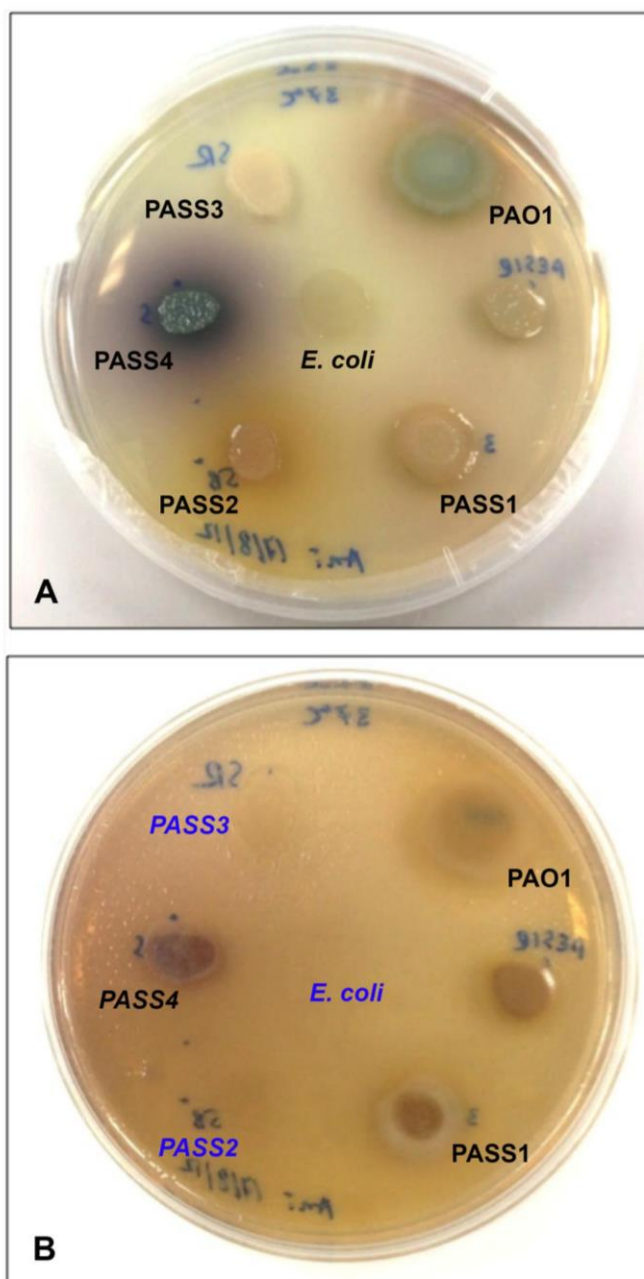
Fig 8. Nematode selective grazing assay using *Caenorhabditis elegans*. A representative Petri dish with colonies of various *P. aeruginosa* isolates before *C. elegans* inoculation (A) and after the 2-week incubation with *C. elegans* (B). Colonies of isolates that have been completely grazed by nematodes, and, thus represent the less-toxic/less-virulent phenotypes, are shown in blue. *Escherichia coli* OP50 was used as a positive control for *C. elegans* grazing.

doi:10.1371/journal.pone.0138527.g008

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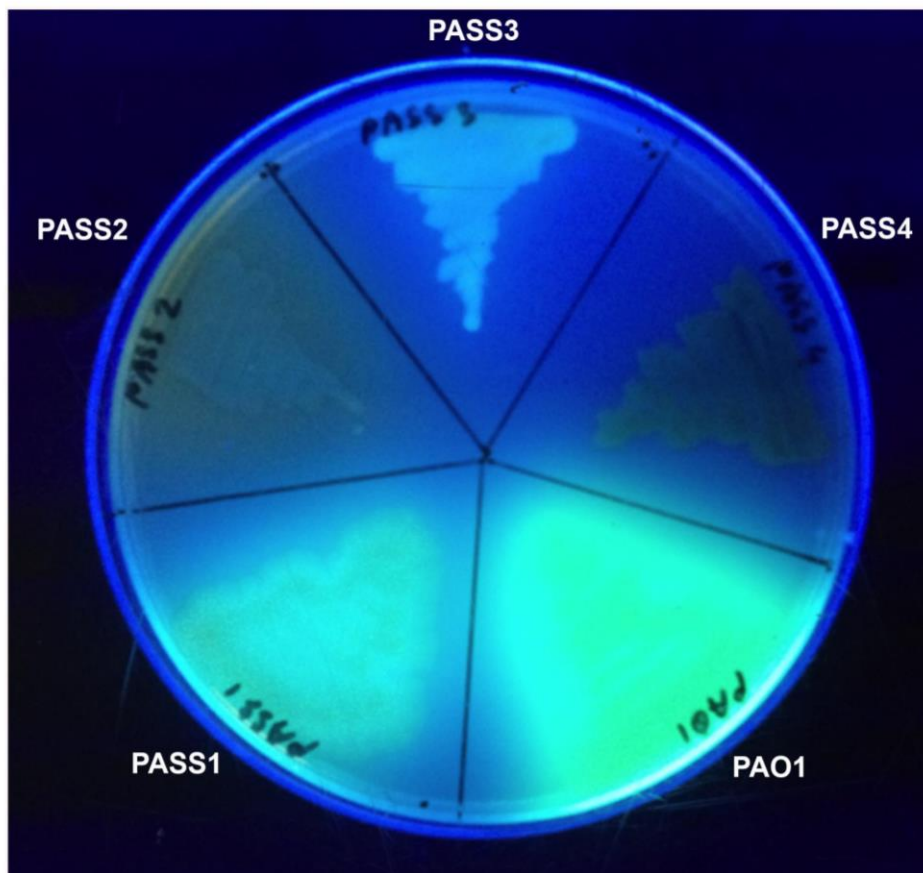
Fig 9. Representative image showing the production of fluorescent siderophore pyoverdine as assessed under the UV light.

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protein PvdJ, and the peptide synthase, respectively; which would explain the lack of pyoverdine production in strains PASS 2 and PASS4. Longitudinal studies of CF patients have reported an increased occurrence of pyoverdine-negative isolates with chronic *P. aeruginosa* colonization [55, 56]. The increased availability of heme in CF lungs may select for the use of hemophores rather than siderophores by *P. aeruginosa* in the CF lung environment [56, 57].

Table 2. Summary of mutations in the *lasR* regulator gene correlated with the observed production of phenazines (PCA, PYO, PCN, 1-OH-PHZ) and pyoverdine (PVD), and the integrity of the pyoverdine biosynthesis gene cluster.

Strain	*lasR* variant	Predicted LasR effect	Production of phenazines	Production of PVD	PVD biosynthesis genomic deficiency (PA2398-PA2402)
PASS1	c.61G>A	p.(A21T)	positive	positive	non-deficient
PASS2	c.693_694insATGGCC	p.M231_A232insMA	negative	negative	deficient
PASS3	c.[61G>A; 124del]	p.[(A21T; D43fs*114)]	negative	decreased	non-deficient
PASS4	No changes	No changes	positive (no PCN)	negative	deficient

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The LasR quorum sensing (QS) regulator has a role in regulation of the production of virulence factors such as phenazines and pyoverdine, and *lasR* mutants have been frequently described among *P. aeruginosa* CF isolates [58, 59]. Sequence analysis of *lasR* in PASS1-4 strains revealed a single nucleotide polymorphism (SNP) in PASS1 and a 6-nucleotide insertion in PASS2 (Table 2). The same SNP observed in PASS1 was also present in the *lasR* gene in PASS3, as well as an additional deletion leading to a frameshift error. The *lasR* gene in PASS4 was identical to that of PAO1. From this analysis, PASS1-2 strains may be attenuated in the LasR regulator, while PASS3 would most certainly be LasR deficient. This may explain the decreased pyoverdine production by PASS3, despite it carrying an intact pyoverdine biosynthetic gene cluster. The inability of PASS2 and PASS4 to produce pyoverdine is almost certainly due to the loss of several pyoverdine biosynthetic genes. The lack of phenazine production by PASS2 and PASS3 might be due to these two strains having the most severe mutations in the *lasR* regulator.

The deficiency in *lasR*, in conjunction with the deficiency in the *psl* cluster of genes responsible for the production of a major exopolysaccharide, may aid to the lifestyle of PASS2 as a potential "social cheater". LasR deficiency may further render PASS2 "deaf" to QS signals and, therefore, limit its response in production of various metabolically costly QS-controlled virulence factors and toxins benefiting the bacterial population [60, 61].

Other virulence factors

Several other virulence factor genes were missing in PASS2 and PASS4, compared to the genome of PAO1. The colicin-like pyocin S5 and its cognate immunity protein (encoded by PA0985 and PA0984 in PAO1 genome respectively), pyocin S4 (PA3866), a probable non-ribosomal peptide synthetase (PA2402), and phospholipase D (PA3487) were absent in both PASS2 and PASS4. In addition, PASS2 also lacks genes for the production of paerucumarin (PA2254-PA2257), chitinase (PA2300), and AMB toxin (PA2302-PA2306).

Distinct *P. aeruginosa* lifestyle strategies in the CF lung

Based on genomic and phenotypic comparisons the four CF isolates obtained directly from the sputum of CF patients revealed significant differences in lifestyle strategy. PASS2 and PASS4 shared some common features with the loss of various virulence determinants, and similar organization of their pili and flagellar loci. PASS2 showed significant genome reduction, with the loss of additional virulence factors, narrowing of potential carbon substrates that can be utilized and even significant reduction in biofilm forming capacity due to the lack of polysaccharide Psl production. The defect in the LasR QS regulator in PASS2 probably also impacts its virulence potential. These adaptations in PASS2 may limit the host immune response as well as reducing expenditure of cellular resources, possibly representing a passive, "cheater" strategy [28].

In contrast, based on the increased production of phenazines such as pyocyanin and increased biofilm forming capacity, isolates PASS1 and PASS4 (the latter even despite the loss of several other virulence genes) may have taken a different adaptation path by enhancing their virulence and colonization potential, possibly representing more aggressive phenotypes. The virulence of PASS1 and PASS4 was also demonstrated against the eukaryotic model *C. elegans* (Fig 8). The virulent phenotype of PASS4 in the *C. elegans* model, despite the clear loss of known virulence factors, highlights an issue in making virulence predictions based on genomic analyses alone.

PASS3 is closely related to PASS1 based on MLST and genomic comparisons, but differs significantly in phenotype. PASS3 displays low toxicity in the *C. elegans* model and is deficient in phenazine production, possibly as a result of mutations in *lasR*. However, PASS3 shows

enhanced biofilm formation and conversion to a mucoid phenotype, which likely enhances its survival and persistence, and therefore may have adopted a more "defensive" strategy.

Table 3 summarizes the overall genetic and phenotypic differences observed between PASS1-4 strains and PAO1. The diversity seen amongst the CF isolates in this and previous studies [62, 63] suggests that there is no single preferred adaptation pathway for success in a CF lung.

In order to enable researchers to broaden *P. aeruginosa* research beyond the limitations of model laboratory strains, recently a *P. aeruginosa* reference panel of 43 strains has been suggested, also based on the origin, in order to reflect its diversity [64]. Nevertheless, the diversity seen among our 4 isolates with each of these strains equipped with a unique set of defensive and offensive tools, suggests that differences that exist between strains that originate even from the same environment like CF can make it impossible to pinpoint a single phenotype/strain with a set of defined characteristics that would be representative for that environment.

CF isolates share a core proteomic signature distinct from PAO1

A shotgun proteomic analysis was undertaken to investigate protein expression profiles of the four clinical isolates and the model *P. aeruginosa* strain. There were striking differences in protein expression between the common laboratory model organism PAO1 and the CF isolates PASS1-4. With ~ 1300–1400 proteins identified for each of these organisms, only 526 were shared among all five, while PAO1 showed 827 unique proteins not detected in any of the CF isolates when cultured in a common laboratory medium LB. Conversely, PASS1-4 strains shared 703 commonly expressed proteins, none of which were detected in PAO1 (Fig 4F, S2 Table). This trend can also be seen on FriPan 2D plot where PAO1 appears at a considerable distance from PASS1-4 strains, while CF strains PASS1-4 among themselves retained the overall similarity profiles with PASS1 and PASS3, and PASS2 and PASS4 clustering together (Fig 4D and 4E), consistent with the overall pattern observed in bioinformatics analysis for all predicted proteins (Fig 4A and 4B).

The proteins uniquely expressed by PAO1 (827 in total) and those uniquely shared by PASS1-4 strains (703 in total), were mapped onto the metabolic pathways of PAO1 using Pathway Tools 17.5 (Fig 10, S2 and S3 Figs). The analysis revealed distinct differences in cell physiology between the CF strains and the model strain PAO1. PAO1 expressed an array of membrane transport proteins involved in the uptake of a broad range of amino acids, polyamines, carbohydrates and other organic nutrients, whose expression was not detected in any of the PASS1-4 strains. There was essentially no expression of biosynthetic genes involved in amino acid, polyamine, carbohydrates, nucleoside or nucleotide biosynthesis (Fig 10A, S2 Fig).

In contrast, PASS1-4 expressed only a small select group of transporters for compounds such as sugars, dipeptides, and heme. Instead the PASS1-4 strains expressed many proteins involved in biosynthetic pathways for compounds such as nucleosides and nucleotides, amino acids, carbohydrates and polyamines (Fig 10B, S3 Fig).

The proteomic data indicates that in a conventional laboratory medium PAO1 may transport a diverse set of "ready-made" nutrients from the rich LB medium, whereas CF isolates PASS1-4 require only a limited number of nutrients from the LB medium, relying mainly on their own metabolism for synthesis of other essential nutrients. Interestingly, despite dramatic differences in the proteins involved in biosynthesis, in general, the expression of proteins involved in the catabolism of various compound groups were similar. This might indicate that PAO1 and PASS1-4 strains all have access to a variety of compound classes, but, as mentioned above, may vary significantly in the means of obtaining these compounds: PAO1 –via transport, and PASS1-4 –via intracellular biosynthesis. This probably reflects specialization in the

Table 3. Summary of genomic and phenotypic characteristics of *P. aeruginosa* CF isolates PASS1-4 and the model strain PAO1.

	PASS1	PASS2	PASS3	PASS4	PAO1
Colony morphology on LB solid medium	pale-green non-mucoid	light brown non-mucoid	white mucoid	green-blue non-mucoid	green non-mucoid
Biofilm formation in flow cells	positive	negative	positive	positive	positive
Flagella	type B	type A	type B	type A	type B
Type IV pili	group II	group I	group II	group I	group II
Swimming motility	positive	deficient	positive	deficient	positive
Binding to mucin	high (80–100%)	low (≤50%)	low (≤50%)	high (80–100%)	medium (50–80%)
Phenazines production	positive	negative	negative	positive (no PCN)	positive
Pyoverdine production	positive	negative	decreased	negative	positive
LasR deficiency	uncertain	possibly deficient	possibly deficient	non-deficient	non-deficient
Loss of additional virulence factors		pyocin S5, pyocin S4, NRPS, phospholipase D, paerucumarin, chitinase, AMD toxin		pyocin S5, pyocin S4, NRPS, phospholipase D,	
Toxicity/ virulence*	positive	negative	negative	positive	positive

* as assessed in *C. elegans* selective grazing assay

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CF strains geared towards utilization of select types of nutrients that might be abundant in CF lungs, such as highly glycosylated mucins, and further highlights the preference for heme as a possible source of iron in CF isolates. Conversely, PAO1, having been grown and maintained in laboratories worldwide for decades, may have adapted to using the broad variety of nutrients present in common growth media, such as LB. The proteomics data reveals significant differences in the metabolic strategies of CF isolates compared with PAO1 and emphasises the limitations presented by the use of laboratory model organisms for studying processes in specific hosts or environments.

Concluding remarks

Significant phenotypic differences were observed amongst the *P. aeruginosa* CF isolates PASS1-4 including differences in traits important for successful CF lung colonization and survival. Phenotypic diversity has been previously shown among the *P. aeruginosa* CF isolates [62, 63], particularly with respect to the decreased virulence in chronic infections [65–67]. While the classical reduction of virulence seen in many CF isolates was observed in PASS2, and the conversion to mucoid phenotype by PASS3, a more aggressive phenotype was shown by PASS1 and PASS4 with increased production of known virulence factors such as phenazines, and increased biofilm formation. This suggests that there is no single pathway of adaptation to an environment such as the CF lung; instead, strains acquire adaptations that allow them to pursue different lifestyles ranging from passive, defensive to aggressive. This makes it extremely difficult to predict the development of certain traits in a particular environment or host, and, hence, make assumptions for the suitable treatment strategies.

Relying on genomic data to make predictions about phenotypes can be problematic. *In silico* analysis of genomes of all 5 isolates in this study revealed a high degree of similarity between the strains with 80% of shared predicted proteome, which is in the range commonly observed for strains in the same species implying possible phenotypic similarities. However, actual protein expression by these strains grown in a common laboratory medium revealed dramatically
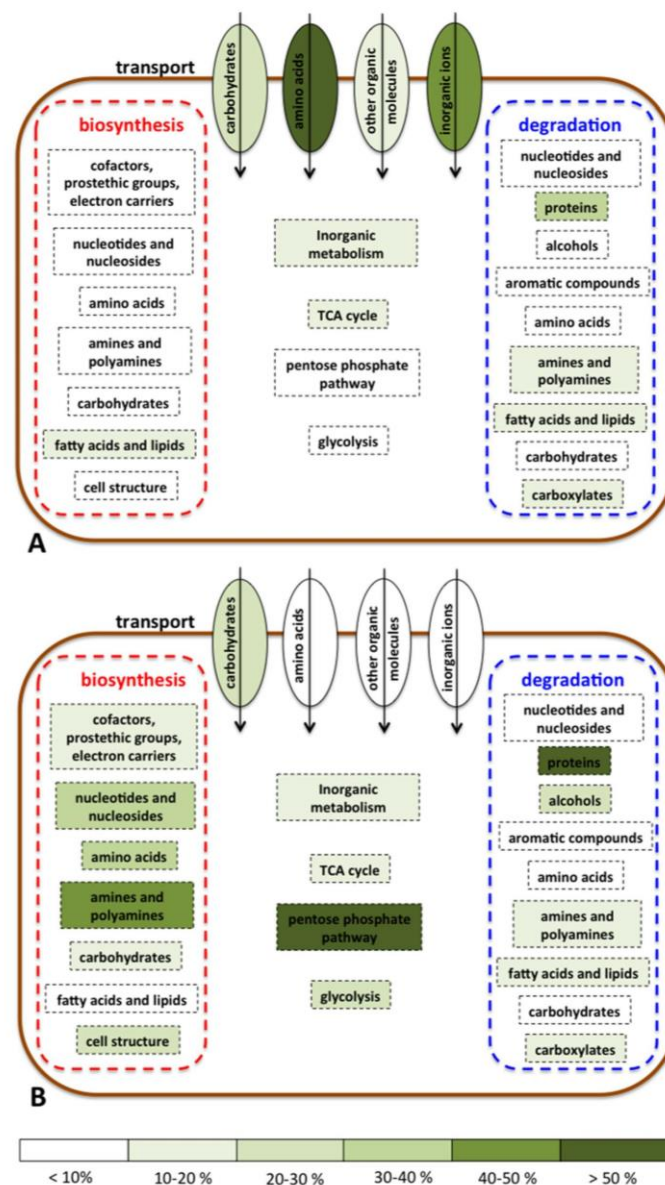
Fig 10. Schematic metabolic overview showing differences in protein abundance between PAO1 (A) and the CF isolates PASS1-4 (B) as assessed via proteomics. Shading represents the percentage of expressed proteins within each metabolic category. Assignment to metabolic categories is based on analysis using Pathway Tools [27]. Full details of the Pathway Tools analysis are shown in S2 and S3 Figs, and the full list of express proteins for each strain is provided in S2 Table.

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different expression profiles suggesting distinct physiological and metabolic states that were not predictable at the genomic level via *in silico* analysis. This further highlights the limitations of model laboratory strains and the need to complement analyses in model organisms with direct experimental work on isolates from the relevant host or environment.

Supporting Information

S1 Fig. Genomic alignment of flagella biogenesis genes in strains PASS1-4 as compared to PAO1 using MAUVE. Sequences conserved among all 5 isolates are presented in mauve, sequences shared between isolates PAO1, PASS1 and PASS3 are in green, sequences shared between isolates PASS2 and PASS4 are presented in blue **(A)**. **Flagella-mediated swimming motility assay for strains PASS1-4 and PAO1 (B).**
(TIF)

S2 Fig. Pathway Tools 17.5 cellular overview diagram showing proteins/pathways expressed uniquely by PAO1, in red. The shapes on the diagram represent specific types of compounds as follows: square—carbohydrate, triangle—amino acid, upside down triangle—cofactor, ellipse (horizontal)—purine, ellipse (vertical)—pyrimidine, diamond—proteins, T-shape—tRNA, circle—all other compounds. The shading of the icon indicates the phosphorylation state of the compound: shaded compounds are phosphorylated; unshaded compounds are unphosphorylated. Shapes/paths located on outer rectangles represent transporters/membrane proteins. The arrows indicate the direction of the transport: pointing in—uptake, pointing out —export.
(TIF)

S3 Fig. Pathway Tools 17.5 cellular overview diagram showing proteins/pathways expressed uniquely by PASS1-4 strains, in red. The shapes on the diagram represent specific types of compounds as follows: square—carbohydrate, triangle—amino acid, upside down triangle—cofactor, ellipse (horizontal)—purine, ellipse (vertical)—pyrimidine, diamond—proteins, T-shape—tRNA, circle—all other compounds. The shading of the icon indicates the phosphorylation state of the compound: shaded compounds are phosphorylated; unshaded compounds are unphosphorylated. Shapes/paths located on outer rectangles represent transporters/membrane proteins. The arrows indicate the direction of the transport: pointing in—uptake, pointing out—export.
(TIF)

S1 Table. MLST allelic profiles and strain types of *P. aeruginosa* isolates obtained from the CF sputum in this study.
(DOCX)

S2 Table. Proteins identified in strains PASS1-4 and PAO1 grown in LB medium via proteomics (p < 0.01). + protein identified in the strain culture (shaded),—protein not identified in the strain culture (unshaded), NA—protein does not have an ortholog in the PAO1 genome or did not map to an ortholog in PAO1 due to differences in the sequence.
(DOCX)

Acknowledgments

Author Contributions

Conceived and designed the experiments: AP SSK KK NHP MPM ITP. Performed the experiments: AP SSK KK AMS VV. Analyzed the data: AP SSK KK AMS CK ITP. Wrote the paper: AP SSK KK AMS CK NHP MPM ITP.

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Chapter-4

Quantitative proteomics by SWATH-MS reflects alterations in the phenotypic profiles of *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients.

Rationale:

The aim of the work described in this chapter was to expand upon the global whole cell quantitative proteome profiling of PASS1-4 strains of *P. aeruginosa*, presented in our published article; Penesyan *et al* (138) (Chapter-3). In this chapter, a comprehensive comparison of protein expression profile of four clinical strains of *P. aeruginosa* PASS1, PASS2, PASS3 and PASS4 is made with the well-known PAO1 strain. Additionally, the differential abundance of proteins in PASS1-4 strains was tested to discern if it reflected the features in the respective genome and phenotypes.

Contribution:

Global whole cell proteome profiles of PASS strains and PAO1 were retrieved from our previously published article (138). I performed SWATH-MS data processing, statistical analysis, data interpretation and writing of the chapter. .

4.1 <u>Background:</u>

P. aeruginosa is a metabolically versatile, opportunistic pathogen and a chief etiological agent in the respiratory tract infections of cystic fibrosis (CF) patients. Life-long colonisation of *P. aeruginosa* in CF lungs, require specific adaptations to encounter stress including nutrients, host immune system effector molecules, toxins from co-inhabiting pathogens, lack of oxygen, antibiotics, among others (140). The bacterium undergoes complex physiological and morphological changes to cope with the challenging micro-environment of the CF lungs (44). For instance, high selective pressure in the CF lungs induces hypermutable phenotype of *P. aeruginosa*, which assist in the generation of diverse, sub-clonal variants, more resistant to the common stresses.

To understand the role of genetic and phenotypic modulations that may assist the bacterium to adapt to the CF lungs, we sequenced the genome and profiled whole cell proteome of four novel strains of *P. aeruginosa* (PASS1, PASS2, PASS3 and PASS4) isolated from the sputum of the CF patients of diverse age groups. Additionally, we performed detailed phenotypic characterization including, biofilm formation, phenazine and pyoverdine production, carbon utilisation, virulence/cytotoxicity against *C. elegans*, motility and mucin binding (152). The study showed that the PASS strains displayed a unique set of genomic modulations which were different to the laboratory strain PAO1 and it was reflected in the phenotypic characteristics. Nevertheless, we observed significant diversity in the phenotypic characteristics between PASS1-4 strains. For instance, PASS2 and PASS4 shared a similar type of flagella (type-A), type IV pili (group-1), and both were deficient in swarming motility, however, PASS4 strain, compared to other strains, imparted a higher level of cytotoxicity against *C. elegans*.

We reasoned, genome modulations contribute largely to the phenotypic nature of each strain, however, protein expression changes could provide additional clues about adaptation dynamics of the PASS strains to the CF lungs. To understand protein expression level differences in the PASS strains, and to explore candidate

proteins which may be specific to cystic fibrosis adapted strains of *P. aeruginosa*, we performed an additional quantitative comparison of proteome profiles of PASS1-4 with PAO1. Specifically, 2D-LC-SWATH-MS quantification data of whole cell proteomes of PASS1-4 and PAO1 were obtained from Penesyan *et al* (152). A quantitative comparison of proteome profiles of PASS1-4 strains was performed by considering PAO1 profile as a baseline. Any changes portrayed by PASS strains in comparison to PAO1 may likely be representative of adaptive strategies used by *P. aeruginosa* in lungs of the CF-affected individuals.

4.2 <u>Materials and Methods:</u>

4.2.1 <u>Bacterial growth conditions:</u>

Bacterial growth conditions have been detailed in the materials and methods section of the Chapter-3 and the published article (138). Briefly, *P. aeruginosa* strains PASS1-4 previously isolated from the sputum of the CF patients (138) and laboratory reference strain PAO1 (ATCC 15692), were grown on solid Luria-Bertani (LB) agar, out of a frozen stock. Colonies of each strain were inoculated in the Luria Bertani (LB) liquid medium and grown aerobically overnight, at 37°C and constant shaking at 150 rpm. Further, cultures of all strains were diluted 1:50 in LB liquid medium and grown at 37°C and constant shaking at 150 rpm till mid-logarithmic phase (O.D at 600nm~6). Cell pellets were collected by brief centrifugation at 2500g for 10 min at 4°C and washed thrice with phosphate-buffered saline (PBS), pH 7.4. The cell pellet was stored in -20^{0}C until use. Bacterial cells were lysed and proteins were separated over SDS-PAGE. Proteins trapped in the gel were digested using trypsin and identified using LC-MS/MS (Triple-TOF 5600, Sciex). Proteins were quantified using SWATH-MS as described in Penesyan *et.al* (138).

4.2.2 <u>Data collection and statistical analysis:</u>

The SWATH-MS quantitative global proteome profile data of *P. aeruginosa* strains, PASS1-4 and PAO1, were retrieved from our previously published original article; Penesyan *et al* (138) as presented in chapter-3. Specifically, cumulative protein areas

of all the quantified proteins of PASS1-4 and PAO1 were exported to a spread sheet for further analysis.

4.2.3   Statistical analysis:

Data processing, normalisation and statistical analysis was performed using Perseus software, version 1.5.2.6 (172). Quantitative values were log-transformed and normalised based on the median value in each sample. Two-sample *t*-tests were performed comparing mean log$_2$, normalised protein area of each protein of PASS1-4 strain against PAO1. A protein was considered differentially expressed if the protein was quantified in all three biological replicates with a difference of log$_2$ mean/fold change of ±1.2 and an FDR adjusted *p*-value <0.01. All profile plots show the mean log$_2$, normalised protein area, while error bars represent the standard deviation. *p*-values are marked by an asterisk in the plots if they are <0.01.

4.2.4   Go annotation:

GO annotation information was manually retrieved from UniProt (http://www.uniprot.org/) using standard PAO1 locus tag identifiers. Corresponding PAO1 locus tag for proteins identified in PASS1-4 strains were obtained from a reciprocal Blast search as detailed in Penesyan *et al* (138).

4.3 Results:

The aim of this study was to establish a quantitative proteomic framework to illustrate differences and similarities between four strains of *P. aeruginosa,* PASS1-4 (PASS strains) isolated from sputum of CF patients. In this light, 2D-LC-SWATH-MS whole cell proteome data presented in Penesyan *et al* (138) was subjected to an additional comparative analysis. Specifically, we compared the quantitative proteome of PASS1-4 with that of laboratory strains PAO1, grown in the planktonic mode in LB broth. The PASS strains used in the study were isolated from CF patients with diverse age, gender, CFTR gene mutation and antibiotic treatment groups and the genomes of the strains were recently reported by us (138). Genome sequencing along with phenotypic assessment provided deeper insights into the vast diversities in the genotype and phenotype, including characteristics in virulence, biofilm

formation, quorum sensing, and pigmentation, which may render survival advantages in the CF host (138).

The data consisted of approximately 1800 accurately quantified protein clusters in each of PAO1 and PASS1-4 strains, across three biological triplicates (Table-4.1 and Supplementary data file 3A and 4A) with >99% confidence (Unused score≥2, peptide FDR <1% (decoy database search)) (138). Reproducibly quantified proteins in this study exceed the previously reported results of the 2D-LC-MS-iTRAQ proteome profiling study of CF isolated transmissible strain AES-1R grown in LB, with 1355 proteins quantified using iTRAQ-MS across the replicates (173). We observed high run-to-run reproducibility across SWATH-MS analysis between the biological replicates (Supplementary Figure-9.4). Interestingly, a large overlap in SWATH-MS quantified proteins was observed in PASS strains (Figure-4F in Penesyan *et al* (138)), compared to PAO1. Of approximately 1800 proteins quantified, 526 were shared between all strains. Strikingly, PASS strains shared 703 proteins, which were not detected in PAO1. Conversely, PAO1 showed 827 unique proteins not detected in PASS1-4 (138). Consistent with this, we previously reported (138), PASS strains isolated from CF patient's sputum shared several phenotypic and genotypic characteristics which were unique compared to laboratory strain PAO1.

Table 4.1: Summary of proteins identified and quantified by SWATH-MS analysis.

Strain	Number of quantified proteins	% of respective ORFs quantified
PAO1	1810	31.8
PASS1	1750	30.2
PASS2	1741	29.7
PASS3	1750	29.9
PASS4	1789	30.1

To gain a deeper understanding of biological functions of quantified proteins, we considered protein expression values of PAO1 and PASS1-4 quantified through the PAO1 strain due to PAO1 standard identifiers, which, unlike novel identifiers of PASS strains, could be used for retrieving gene ontology (GO) functional information. GO information was retrieved from UniProt (http://www.uniprot.org/) for three categories; 1) molecular functions (1212 proteins) 2) cellular components (835 proteins) and 3) biological process (1285). A large proportion of proteins (~50%) under molecular functions were known to be involved in the catalytic activity (848 proteins) and binding (699 proteins) (Figure-4.1). 416 proteins were assigned as membrane proteins accounting for 32% of identified proteins. The largest class of proteins under biological processes included proteins designated, metabolic process which included 1071 proteins (60%).
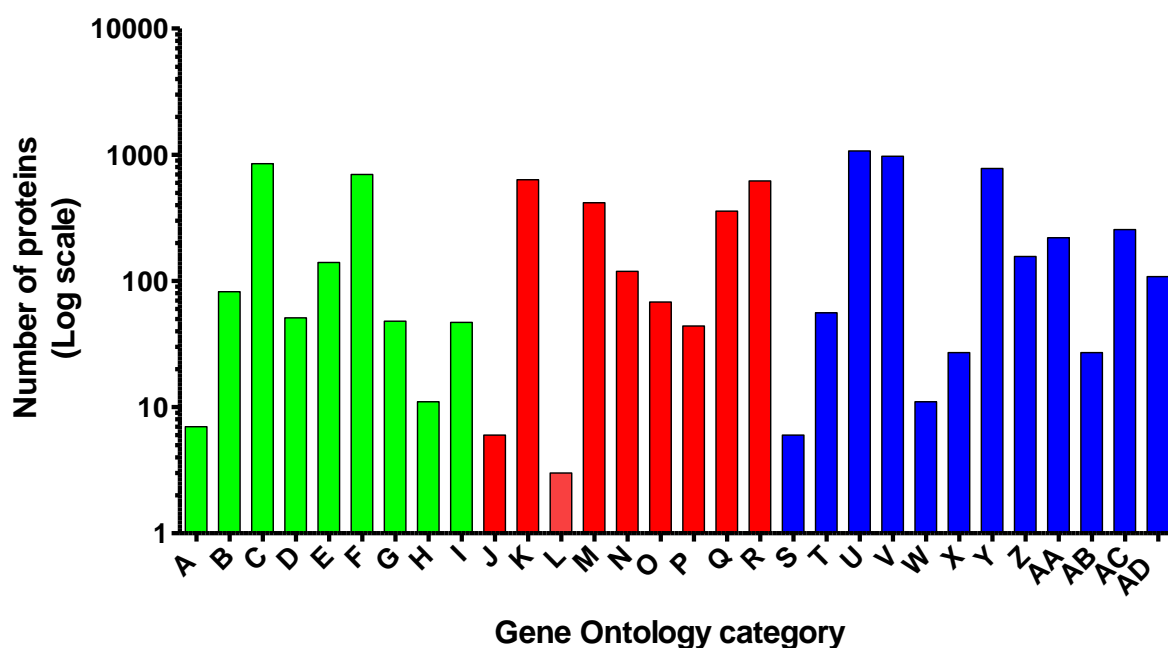
Figure-4. 1: Gene ontology (GO) categories of *P. aeruginosa* proteins quantified in SWATH-MS analysis: Green, red and blue bars represent molecular functions, cellular component, and biological process respectively with 1212, 835 and 1285 representative proteins respectively. The GO categories are; (A) transcription factor activity, protein binding (B) transcription factor activity, sequence-specific DNA binding (C) catalytic activity (D) structural molecule activity (E) transporter activity (F) binding (G) electron carrier activity (H) antioxidant activity (I) molecular transducer activity (J) extracellular region (K) cell (L) nucleoid (M) membrane (N) macromolecular complex (O) organelle (P) organelle part (Q) membrane part (R) cell part (S) reproduction (T) signal transduction (U) metabolic process (V) cellular process (W) antioxidant activity (X) locomotion (Y) single-organism process (Z) response to stimulus (AA) localization (AB) multi-organism process (AC) biological regulation (AD) cellular component organization or biogenesis.

4.3.1 Comparing protein expression between PASS1-4 and PAO1 strains

We performed Student's *t*-tests comparing expression profiles of individual PASS strains with PAO1. After applying a stringent $\log_2$ fold change cut-off of ±1.2, and an adjusted *p*-value cut-off of $p \leq 0.01$, we identified 1230 differentially expressed proteins, collectively in PASS1-4, which were consistently identified across three biological replicates (Supplementary Figure-9.5). 65 proteins were differentially expressed in all PASS strains (Figure-4.2) of which 41 proteins had a similar trend of expression, either up or down regulated in all the PASS strains. The largest number of differentially expressed proteins were observed in PASS2 (655 proteins, with 189 differentially expressed proteins exclusive to PASS2) and the least were observed in

PASS1 (453 proteins). This is consistent with features observed in phenotype characterization and genome analysis, where PAO1 and PASS1 shared many phenotypic characteristics including, green pigmentation on LB plates, type-B flagella, virulence, phenazine and pyoverdine production and similar biofilm morphology (Table-3 in Penesyan *et al* (138)). On the other hand, PASS2 is quite distinct and differs to that of PAO1 in biofilm formation, flagellin type, pigmentation and virulence. Thus, phenotype and genome level differences between PAO1 and PASS strains were in part reflected in differential protein expression profiles.
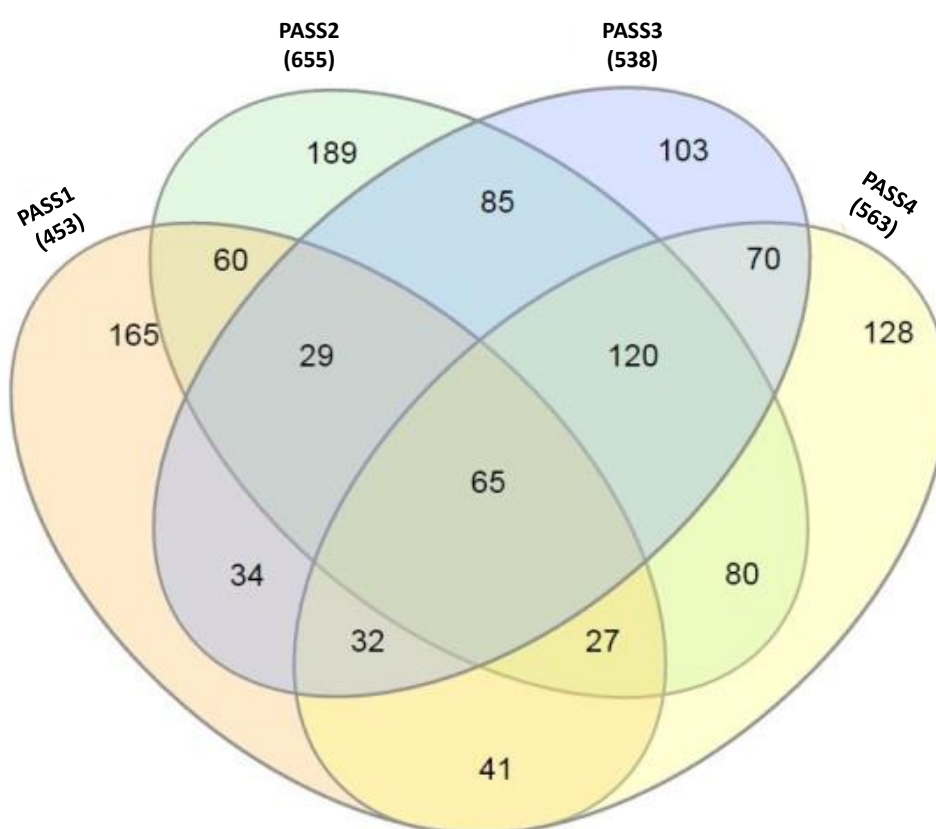
Figure-4.2: Features of differentially expressed *P. aeruginosa* proteins. Venn diagram depicting unique and common differentially expressed proteins between PAO1 and PASS1-4 strains.

4.3.2 Phenazine production in PASS strains is heterogeneous but reflects the phenotype:

Phenazines are biologically active, small diffusible metabolites produced by bacteria including *P. aeruginosa* that function as broad-spectrum antibiotics. They are potent virulence factors against an array of hosts including microorganisms and mammals (174, 175). *P. aeruginosa* produces four types of phenazines including pyocyanin, phenazine-1-carboxamide (PCN), 1-hydroxyphenazine (1-OH-PHZ) and phenazine-1-carboxylic acid (PCA) (174). Biosynthesis of all phenazines initiates from chorismate and a four-step enzymatic process generates a common intermediate molecule, phenazine-1-carboxylate, which can be enzymatically converted to various phenazines (175, 176).

Through phenotypic assays, we previously illustrated the absence of phenazine production in PASS2 and PASS3 and increased production in PASS1 and PASS4 in comparison to PAO1 (138). Through SWATH-MS quantification, we detected seven proteins, PA4210-PA4216, from one of two operons (operon name: *phzB1-phzC1-phzD1-phzE1-phzF1-phzG1*) involved in the phenazine biosynthesis. These proteins enzymatically aid synthesis of phenazine-1-carboxylate through chorismate (177). PhzA1, PhzC1, PhzE1 and PhzG1 (PA4210, PA4212, PA4214 and PA4216) were down-regulated in PASS2-3 in comparison with PAO1. Conversely, PhzA1, PhzB1, PhzC1, PhzF1 (PA4210, PA4211, PA4212 and PA4215) were increased in abundance in PASS1 and PASS4 in comparison with PAO1 (Figure-4.3). For instance, protein PhzA1(PA4210), involved in the first step of biosynthesis of phenazines converting chorismate to 2-amino-4-deoxyisochorismate, was reduced in expression by two-fold in PASS2-3 and increased in expression by two-fold in PASS1 and PASS4 in comparisons to PAO1(Figure-4.3).

Reduction of abundance of phenazine biosynthesis proteins in PASS2-3 might be one of the contributor to the reduced cytotoxicity of PASS2-3 towards *C. elegans* as demonstrated in a grazing assay (138).
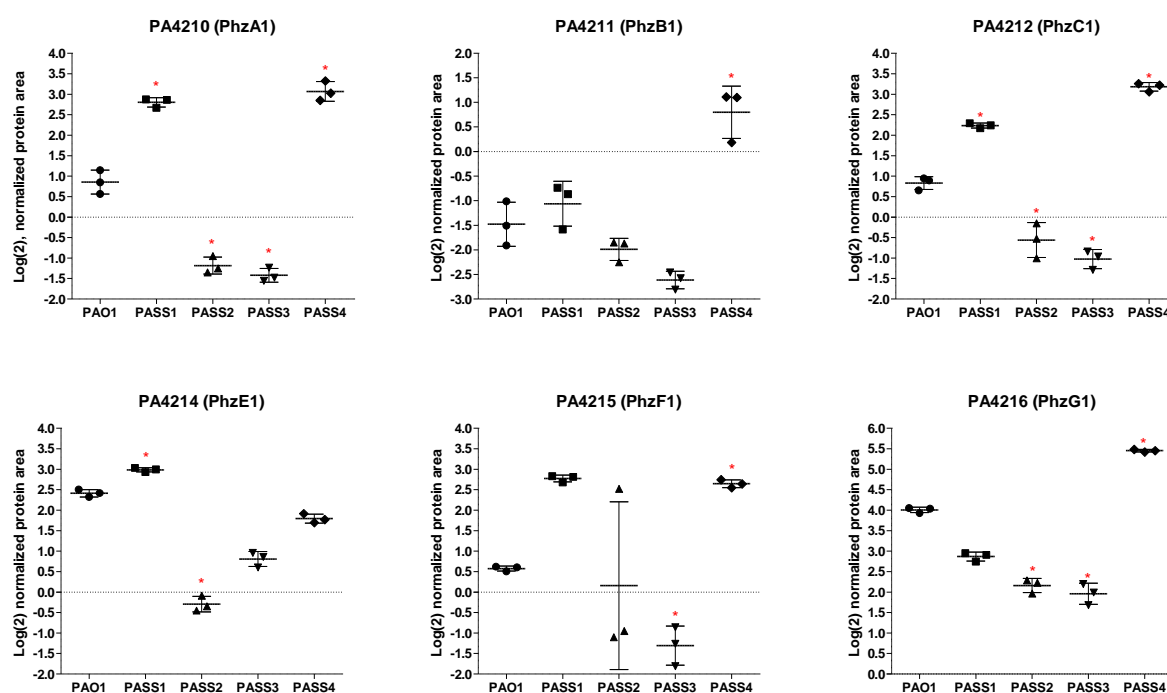
Figure-4.3: Differential plot of protein areas of *P. aeruginosa* phenazine biosynthesis proteins. Each point represents the $\log_2$ transformed and normalised protein area of each replicate in all PASS strains and the vertical bars represent the standard deviation. Red asterisks represent statistically significant, differentially expressed proteins in comparison to PAO1 (Student's *t*-test *p*-value<0.01, fold change±1.2).

4.3.3 PASS strains portray reduced expression of several aerobic respiration proteins:

The *P. aeruginosa* respiratory chain is relatively complex compared to other Gram-negative bacteria (6). It consists of an array of electron donors and acceptors that include 17 primary dehydrogenases, ubiquinones, b and c-type cytochromes and five terminal oxidases (6), all of which collectively generate a proton gradient utilised by ATPase to produce ATP. We quantified several proteins involved in aerobic respiration and strikingly, the majority of the proteins were decreased in abundance in PASS1-4 strains when compared to PAO1.

Interestingly, the majority of primary respiratory dehydrogenases, responsible for feeding electrons from respiratory substrates into the quinone pool were down-regulated in PASS1-4 strains compared to PAO1 (Supplementary data file 4A, Figure-4.4). For instance, PASS1-4 showed decreased abundance of the majority of

quantified NADH-dehydrogenase-1 subunits (NuoA, NuoB, NuoD-NuoN) when compared to PAO1 (Figure-4.4A).

NADH dehydrogenase I

Other dehydrogenases
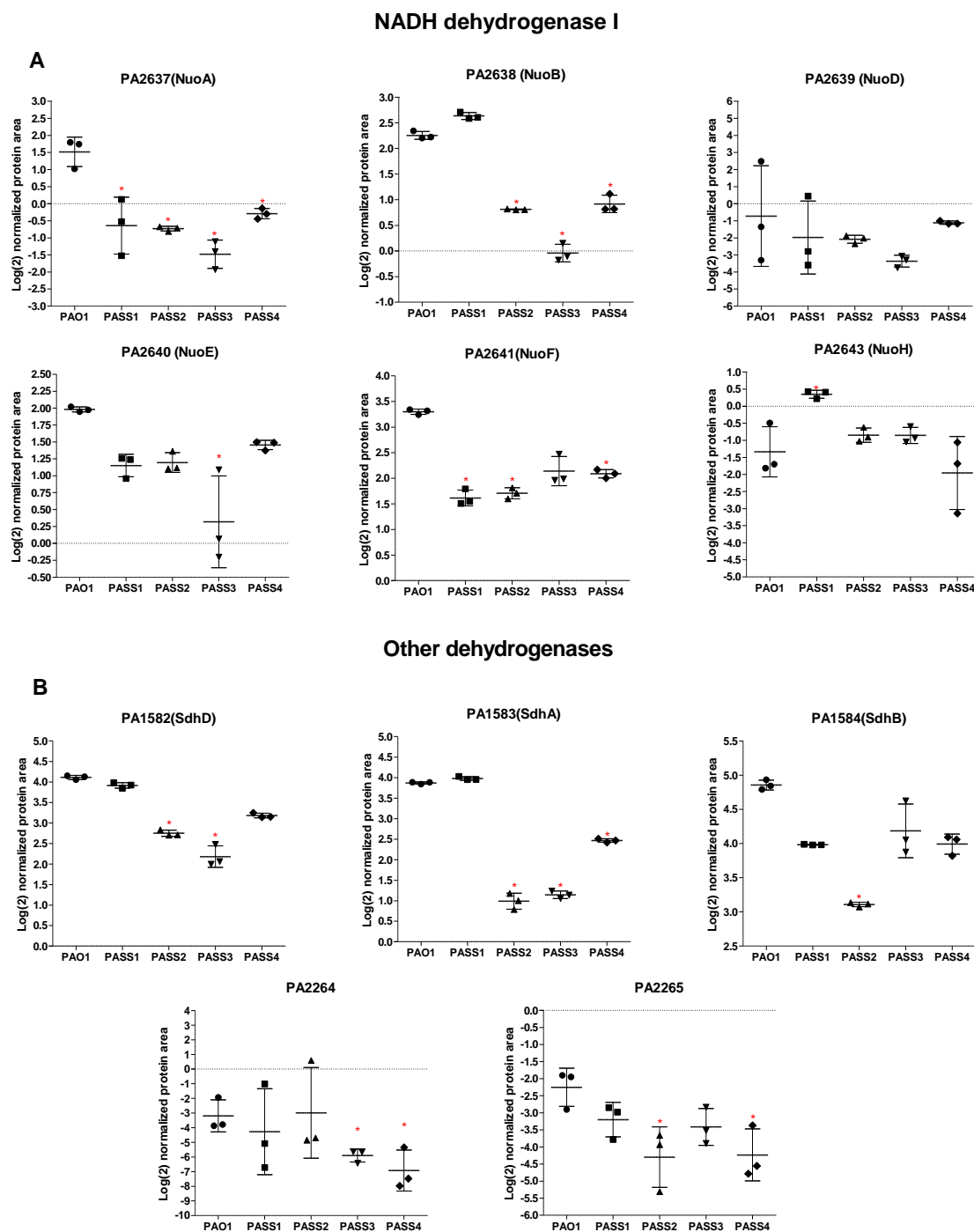
Figure-4.4: Differential plot of protein areas of respiratory dehydrogenases involved in electron transport chain of *P. aeruginosa*. Differential plots of subunits of (A) NADH dehydrogenase-1and (B) succinate dehydrogenase (SdhA,B,D) and Gluconate 2-dehydrogenase (PA2264-PA2265). Each point represents the $\log_2$ transformed and normalised protein area of each replicate in all PASS strains and the vertical bars represent the standard deviation. Red asterisks represent statistically significant, differentially expressed proteins in comparison to PAO1 (Student's *t*-test *p*-value<0.01, fold change±1.2).

Other quantified dehydrogenases included succinate and gluconate dehydrogenase. Of four known subunits of succinate dehydrogenase (SdhA, SdhB, SdhC and SdhD), we identified three in PASS1-4 and PAO1 and two or more subunits were 1.3 to 2.8 fold decreased in abundance in PASS2-PASS4, while, expression of all subunits in PASS1 was similar to PAO1 or not significantly altered (Figure-4.4B).

Ubiquinone is the main quinone in *P. aeruginosa*, aiding in transferring of electrons from primary dehydrogenases either to the cytochrome bc1 complex or directly to quinol oxidases (6). There are six predicted ubiquinone biosynthesis genes (*ubiA, ubiB, ubiC, ubiE, ubiG and ubiH*) in *P. aeruginosa*, of which, UbiA protein was down-regulated in PASS2-4 and UbiE was down-regulated in PASS3 in comparison to PAO1. Additionally, three proteins (PA4428-PA4431) of the cytochrome *bc1* complex were quantified and all the proteins were decreased in abundance in PASS2-4 in comparison to PAO1. Further, CcmH (PA1482) protein involved in the cytochrome biosynthesis was down-regulated in PASS2 and PASS3 in comparison to PAO1. Lastly, cytochrome C1 precursor (PA4429) and probable cytochrome C (PA2482) showed much less *n*-fold change in two or more PASS strains compared PAO1 (Supplementary data file 4A).

In the final step of ETC of aerobic respiration, terminal oxidases (the equivalent of Complex IV of eukaryotes) reduce oxygen (O_2) to water (H_2O). Five terminal oxidases are encoded by *P. aeruginosa* genome which are tightly regulated depending on the availability of oxygen and surrounding environment (178). *Cbb3* oxidases (*cbb3*-1 and *cbb3*-2) are known to have high affinity towards oxygen, making them key players in the respiration of *P. aeruginosa*, more so during hypoxic conditions (6, 120). *Cbb3*-1 and *Cbb3*-2 oxidases are encoded by *ccoN1O1Q1P1* (PA1552–1554) and *ccoN2O2Q2P2* (PA1555–1557) operon respectively (120).

Strikingly, all of the five subunits of *cbb3* oxidases quantified in this study were down-regulated in PASS1-4 compared to PAO1 (Figure-4.5). Intriguingly, expression of cyanide-insensitive oxidase, CioA was elevated in PASS2 and PASS3, however, the protein was not significantly altered in PASS1 and PASS4 when compared to PAO1

(Supplementary data file 4A). Considering the known induction of CioA under an elevated concentration of cyanide (179), confoundingly, HcnAB, involved in the biosynthesis of cyanide in *P. aeruginosa*(180), was decreased in abundance in PASS1-3 in comparison to PAO1. Lastly, bo_3-type (Cyo) and aa_3 oxidases, major players in the respiration of *P. aeruginosa* under oxygen abundant condition (178), were not detected in our study.
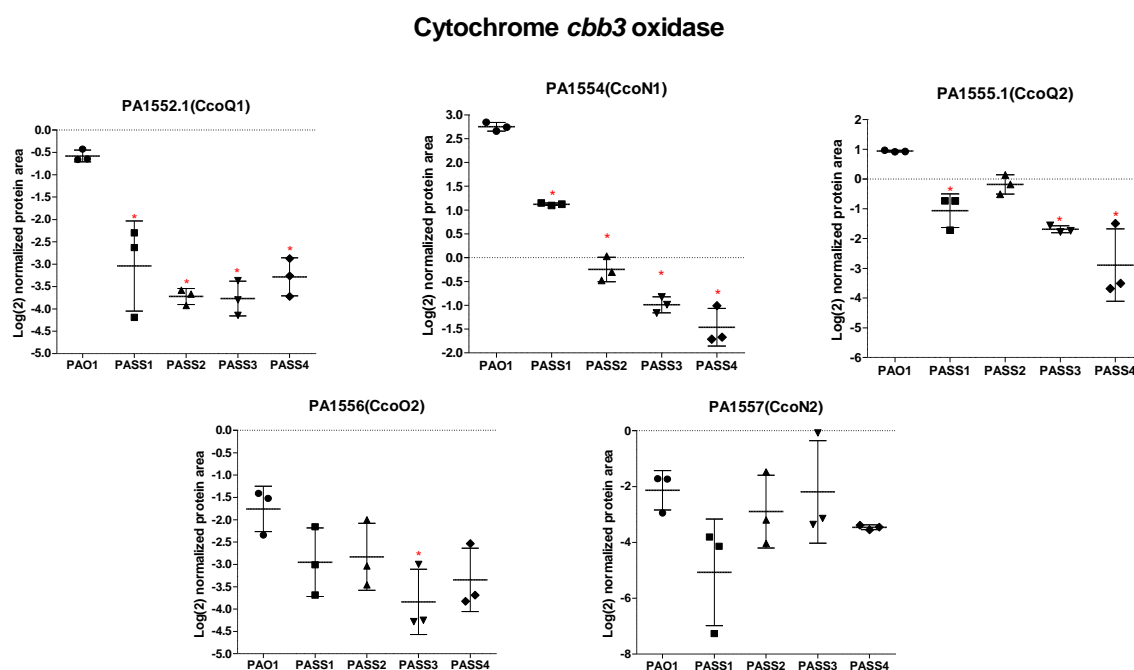
Figure-4. 5: Differential plot of protein areas of *P. aeruginosa* Cytochrome *cbb₃* oxidase involved in electron transport chain. Each point represents the log$_2$ transformed and normalized protein area of each replicate in all PASS strains and the vertical bars represent the standard deviation. Red asterisks represent statistically significant, differentially expressed proteins in comparison to PAO1 (Student's *t*-test *p*-value<0.01, fold change±1.2).

Although, the bacterial cells were grown aerobically for this experiment, expression of several proteins involved in anaerobic respiration was observed in all the strains. We quantified 16 proteins (Supplementary data file 4A) involved in anaerobic respiration through dissimilatory denitrification and arginine fermentation in *P. aeruginosa* and we observed a heterogeneous pattern of expression between PASS strains. For instance, nitrate reductase subunits NarJ and H were increased in abundance exclusively in PASS1 and unaltered in PASS2-4 when compared to PAO1. On the other hand, NirC, which mediates electron transfer from the cytochrome bc1

complex to nitrite reductase, was decreased in abundance in all PASS strains, compared to PAO1. Activation of a few denitrification enzymes in PASS strains might have been caused in part due to high cell densities during exponential growth, leading to relatively less availability of oxygen. Consistent with our findings, the coexistence of microaerobic respiration and nitrate respiration was previously shown in *P. aeruginosa* PAO1 (128, 181).

P. aeruginosa ATPase is synthesised by *atpI-atpB-atpE-atpF-atpH-atpA-atpG-atpD-atpC* operon. We quantified alpha, beta, gamma, delta and epsilon (PA5553-PA5561) chains of the F0 subunit of ATPase enzyme. Additionally, we quantified C-chain (PA5559) of the F1 unit. Following the trend of down-regulation of proteins associated with ETC, the majority of subunits of the ATPase were decreased in abundance in two or more PASS strains in comparison to PAO1 (Figure-4.6).

ATP synthase complex
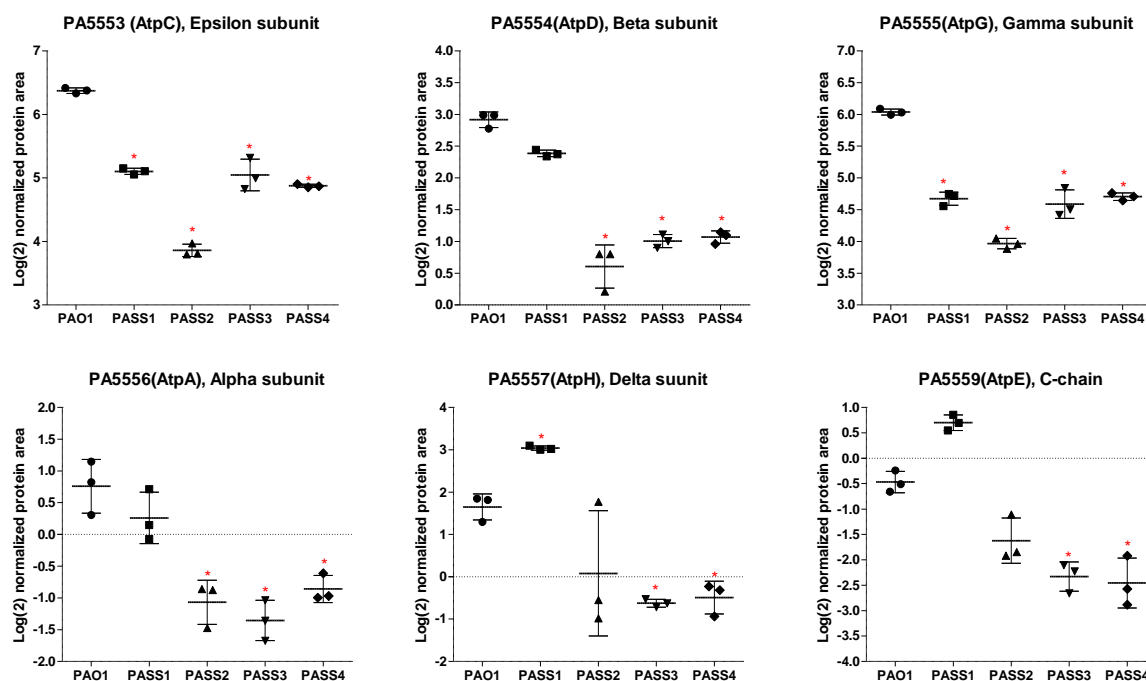
Figure-4.6: Differential plot of protein areas of subunits of *P. aeruginosa* ATP synthase enzyme involved in electron transport chain. Each point represents the log$_2$ transformed and normalised protein area of each replicate in all PASS strains and the vertical bars represent the standard deviation. Red asterisks represent statistically significant, differentially expressed proteins in comparison to PAO1 (Student's *t*-test *p*-value<0.01, fold change±1.2).

To summarise, we quantified several proteins associated with the aerobic and anaerobic respiration, including primary dehydrogenases, ubiquinone biosynthesis, cytochrome *bc1* complex, terminal oxidases, reductases and energy generation through ATPase in PASS1-4 and PAO1. A large proportion of the proteins were decreased in abundance in PASS strains in comparison to PAO1. Although we observed a heterogeneous pattern of expression of several denitrification proteins, a few were increased in abundance in PASS strains, including NarJ, NarH. These results may indicate decreased rate of aerobic respiration of PASS strains in comparison to PAO1.

4.3.4 Type VI secretion system (T6SS)

P. aeruginosa is reported to have three kinds of type VI secretion system (T6SS), H1-T6SS to H3-T6SS (182). Interestingly, the majority of the H1-T6SS proteins were increased in abundance almost exclusively in PASS4 but not in PASS1-3 in comparison with PAO1 (Figure-4.7). Three proteins, Hcp1, TssB1 and TssC1, which are parts of injector assembly of the secretion system, were increased 2.2, 2.1 and 1.4 fold respectively in PASS4, and not significantly altered in PASS1-3, when compared to PAO1. Another notable conserved T6SS associated protein, ClpV (183) was increased 1.7 fold in expression exclusively in PASS4. Of note, an amidase type of toxin, Tse1 (PA1844) which acts on peptidoglycan of rival cells and degrades it, was increased in expression in PASS2-4 by 1.7 to 3.5 fold. Interestingly, this was the only protein of T6SS, which showed an increased *n*-fold change in almost all PASS strains (PASS2-PASS4) except PASS1. Tse6 toxin was recently reported to act on target cells by deteriorating the universally essential cellular components, NAD+ and NADP+ (184). However, there was no significant observable change in expression in PASS strains compared to PAO1. In line with an increased abundance of T6SS proteins in PASS4, we observed 1.7 fold increase in abundance of Fha1 in PASS4, a forkhead-associated domain protein which regulates the activity of the T6SS by changes in the phosphorylation state by a transmembrane serine-threonine Hanks-type kinase, PpkA (183). However, an ortholog of PpkA was absent in PASS4 genome, which poses a question what regulates the activity of Fha1 protein.

In summary, up-regulation of T6SS proteins almost exclusively in PASS4, and down-regulation of H2-T6SS proteins in PASS2-3 may suggest PASS4 may portray better T6SS mediated virulence in comparison to PASS1-3 and PAO1. Consistent with this, phenotypic assays (138), showed features that are often indicative of better virulence, including thick biofilm formation, increased binding to mucins and increased toxicity against *C. elegans.* Contrastingly, PASS2-3 showed decreased motility and cytotoxicity against nematode *C. elegans*.

Type six secretion system (T6SS)
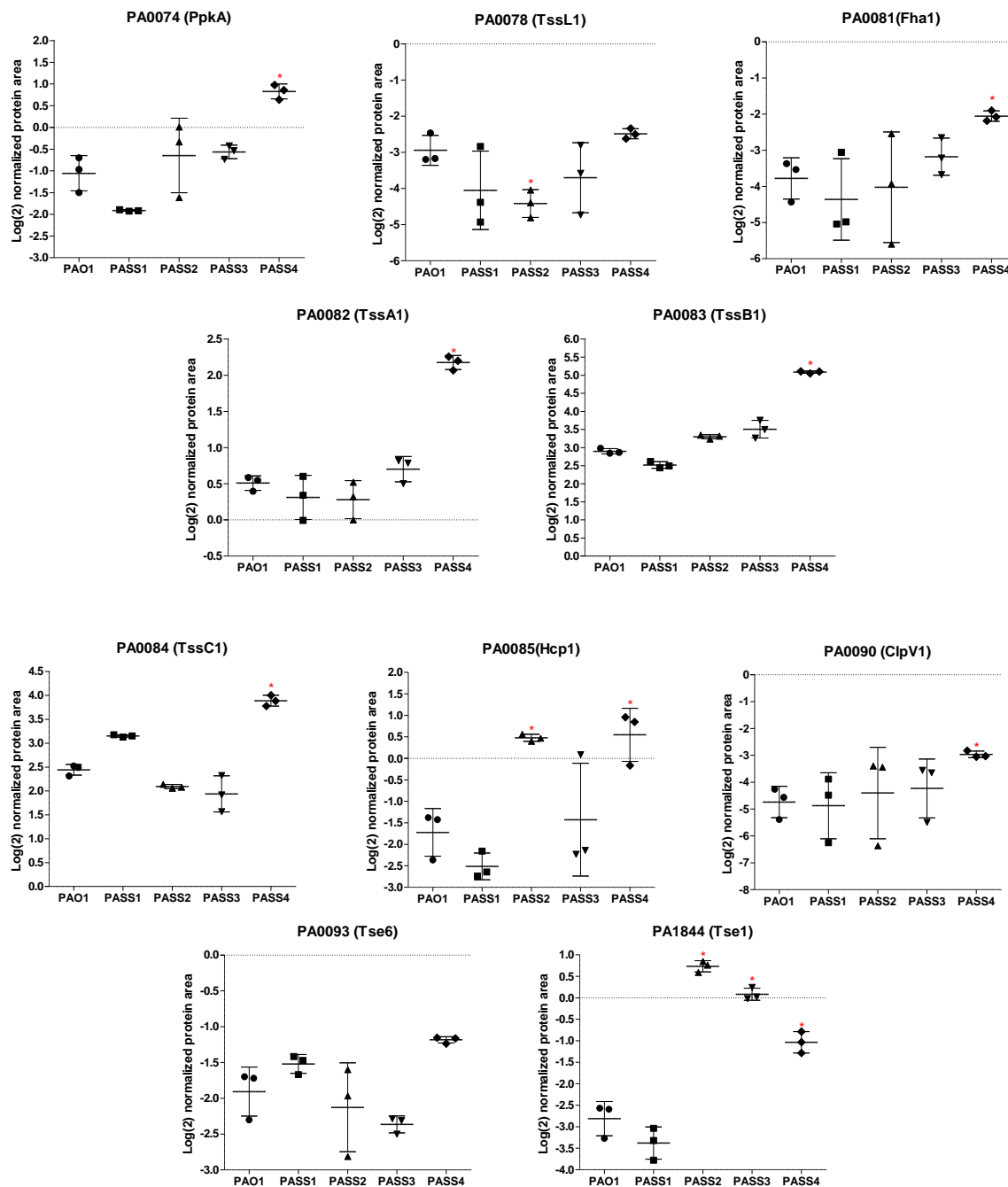
Figure-4. 7: Differential plot of protein areas of protein components of T6SS. Each point represents the $\log_2$ transformed and normalised protein area of each replicate in all PASS strains and the vertical bars represent the standard deviation. Red asterisks represent statistically significant, differentially expressed proteins in comparison to PAO1 (Student's *t*-test *p*-value<0.01, fold change±1.2).

4.3.5 Differential expression of PQS

P. aeruginosa produces 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS), a quorum sensing (QS) molecule that has been shown to induce the expression of virulence factors (185, 186). The synthesis of PQS requires enzymes encoded by the *pqsABCDE, phnAB*, and *pqsH* operon (Figure-4.8A). Products of *pqsABCD* genes, synthesise PQS from anthranilate and the keto-fatty acid and form an intermediate molecule 2-heptyl-4(1H)-quinolone (HHQ), which is ultimately converted into PQS by PqsH (PA2587) (187, 188).

We quantified a panel of six proteins (Figure-4.8B) involved in the biosynthesis of PQS. Interestingly, the majority of them were upregulated in PASS4, decreased in abundance in PASS2-3 and unaltered or not significantly changed in expression in PASS1, compared to PAO1. For instance, we detected ~2 fold up-regulation and 1.5-2 fold down-regulation of PqsABCD in PASS4 and PASS2-3 respectively. Further, MvfR (PqsR) which positively regulates the HAQ biosynthesis (189), was elevated in expression in PASS4 by 1.5 fold. Additionally, MvfR was also upregulated in PASS2, however, changes in expression of downstream proteins controlled by MvfR was not observed (Figure-4.8B). Interestingly, *lasR* regulator, which is a positive regulator of *pqsABCDE* (190), was mutated in both PASS2 and PASS3 (138), and the *lasR* gene of PASS4 was not mutated and was identical to that of PAO1. This might explain the reduced production of PQS in PASS2 and PASS3. Increased expression of PQS biosynthesis proteins in PASS4 and decreased expression in PASS2 and PASS3 might contribute to the increased and decreased cytotoxicity against *C. elegans* in PASS4 and PASS2-3 respectively, as reported previously (191, 192).
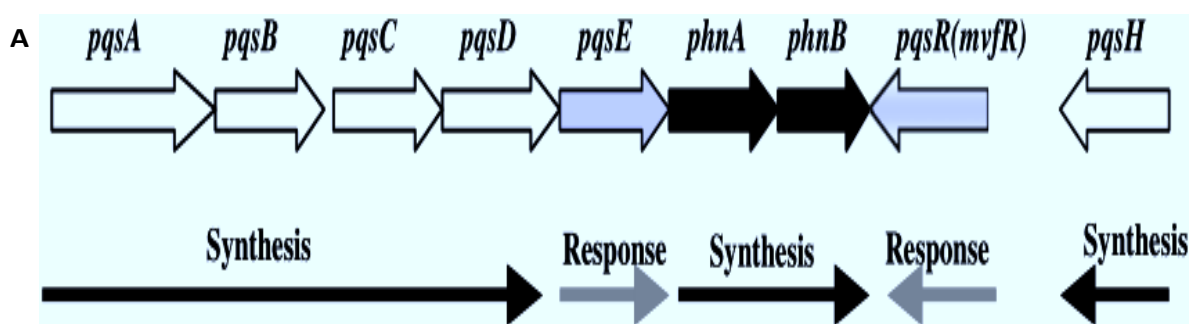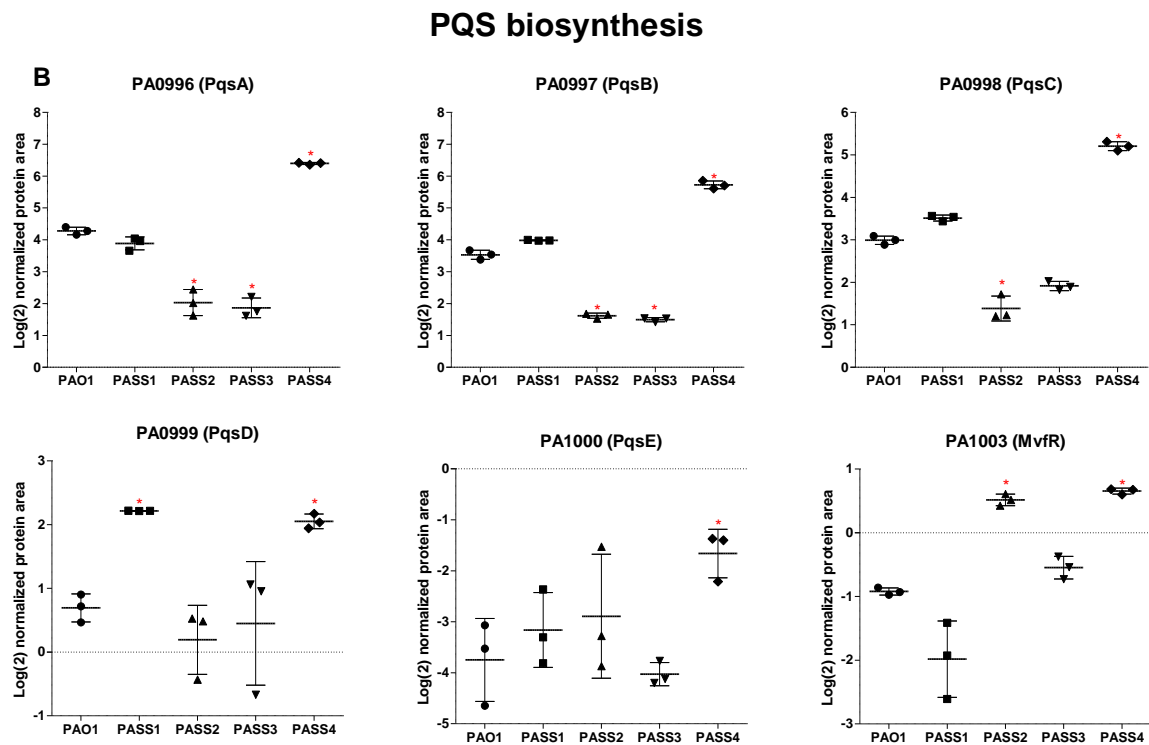
PQS biosynthesis

Figure-4. 8: PQS biosynthesis in *P. aeruginosa*. (A) The products of *pqsABCDE* and *phnAB* operons drive the biosynthesis of HHQ a precursor of PQS and these genes are independently regulated by the PqsR (MvfR). PqsH converts the PQS precursor HHQ into PQS. (B) The differential plot of protein areas of PQS biosynthesis and regulatory proteins. Each point represents the $\log_2$ transformed and normalized protein area of each replicate in all PASS strains and the vertical bars represent the standard deviation. Red asterisks represent statistically significant, differentially expressed proteins in comparison to PAO1 (Student's *t*-test *p*-value<0.01, fold change±1.2).

4.3.6   Other virulence factors:

Consistent with the notion that the PASS2 and PASS3 have reduced virulence in comparison to PASS4 and PAO1 (as demonstrated by *C. elegans* cytotoxicity assay by Penesyan *et al*(138)), we observed decreased abundance of proteins associated with hydrogen cyanide (HCN) synthesis, HcnA (PA2193), HcnB (PA2194) in PASS1-3 in comparison with PAO1, however, levels in PASS4 were similar to that of PAO1. Interestingly, another virulence determinant, alkaline metalloproteinase (AprA), secreted by Type-1 secretion system (T1SS) was reduced in abundance by ~2 fold in PASS2-3 and not significantly changed in PASS1 and PASS4. Unsurprisingly, reduced expression of HCN synthesis proteins and AprA might be two of the many

factors contributing towards reduced toxicity of PASS2-3, compared to PAO1, towards *C. elegans*.

4.4 <u>Discussion:</u>

Agents that help *P. aeruginosa* in pathogenesis, provide a survival advantage and harm the hosts' cellular structure, include a diverse battery of metabolites that are synthesised by sensing the surrounding niche. One such virulence determinant is phenazine which has been detected in the CF sputum (Pyocyanin up to 27.3 μg/ml and 1-Hydroxyphenazine up to 5.2 μg/ml) (193). The detected amount of phenazines positively correlated with the population density of *P. aeruginosa* and negatively correlated with host lung function (194), thus underscoring its significance in the pathogenesis of *P. aeruginosa* in CF lungs.

The majority of the proteins involved in the biosynthesis of phenazines detected in our study were increased in expression in PASS1 and PASS4, on the contrary, it was decreased in PASS2-3. Strikingly, these results correlate well with our previous observation performed through UHPLC measurement of purified phenazines from PASS strains, where PASS1 and PASS4 produced phenazines, and PASS2-3 showed no measurable traces.

Phenazine production has been linked to acute and chronic *P. aeruginosa* CF lung infections (195). They are known to cause tissue destruction through oxidative stress, depletion of cAMP, ATP, and major antioxidant, glutathione (177). Additionally, they have been demonstrated to contribute to the acquisition of iron, serve as intercellular signals and affect biofilm architecture (194). Notwithstanding, while it seems to be advantageous to maintain such virulence determinants, mutation and reduced production of phenazines in *P. aeruginosa* have been reported previously (69). An independent study showed the reduced production of the phenazine, pyocyanin, in PAO1 and PA14 strains due to a mutation in biosynthesis genes which ultimately resulted in attenuation of the bacterium in both acute and chronic mouse lung infection models (89). Down-regulation of phenazine biosynthesis proteins in PASS2 and PASS3, would have been caused by a severe mutation in *lasR* regulator in PASS2

and PASS3, which is one of the key regulators of the phenazine production in *P. aeruginosa* (138).

Secretion systems in *P. aeruginosa* are prime vehicles that deliver the virulence determinants during the course of the pathogenesis (196). T6SS comprises a structure reminiscent of the inverted T4 bacteriophage type of hexameric haemolysin co-regulated protein 1 (Hcp1) assembly, connected to a puncturing device formed by valine-glycine repeat protein G1 (VgrG1). This contractile injection apparatus is capable of penetrating the target membrane and delivering virulence effectors into the eukaryotic host or a competing bacterium (197). Of note, non-pathogenic bacteria are also shown to have loci encoding T6SSs, which might suggest, induction of T6SS expression may not be restricted to an intimate interaction of bacteria with the host (198, 199).

Interestingly, PASS4, almost exclusively displayed an increased expression of the majority of proteins in relation to H1-T6SS, while expression in PASS1-3 were similar to PAO1. Mougous *et al* (197) detected Hcp1 protein, a component of T6SS, in the sputum of the CF patients with *P. aeruginosa* airway infection, however, it was undetected in the sputum of the uninfected individuals (197). This underscores the importance of T6SS in pathoadaptation of the bacterium in a polymicrobial infection such as CF airway infection. Apart from virulence, components of T6SS such as TssC1, are shown to contribute towards antibiotic resistance in biofilms (199, 200). Hence, it is likely that up-regulation of T6SS related proteins may provide a survival advantage to *P. aeruginosa* during the establishment of CF airway infection.

Hydrogen cyanide (HCN) is one of the many virulence factors secreted by *P. aeruginosa* to curb the growth of competitor microbes and cause damage to the host tissue. HCN is highly toxic and easily diffusible through tissues and binds irreversibly to the terminal oxidases of respiratory chain to halt aerobic respiration (180). *P. aeruginosa* produces HCN at concentrations up to 300 μM under low oxygen conditions (120), however, it avoids the effects of autogenic HCN by inducing higher expression of cyanide-insensitive terminal oxidase (PA3929 and PA3930) in the

respiratory chain (180). Virulence potency of *P. aeruginosa* HCN was demonstrated in *C. elegans* infection model, where HCN causes paralytic killing of *C. elegans* (191). Clinical significance of HCN in lung infection was demonstrated by the detection of HCN in the sputum and the breath of CF and non-CF bronchitis patients with known *P. aeruginosa* infection. However, it was absent in non-*P. aeruginosa* infected bronchitis patients and healthy counterparts (180), indicating *P. aeruginosa* is the major producer of HCN. Hence, there is interest in using HCN as a surrogate marker for the rapid detection *P. aeruginosa* infections in the respiratory diseases.

Following the notion of decreased virulence in PASS2-3, HCN biosynthesis proteins (HcnA and HcnB) were decreased in abundance in PASS1-3 and remained unaltered in PASS4 with reference to PAO1. Reduced production of HCN in PASS2-3 may be one of the reason for the reduced cytotoxicity of the bacterium against *C. elegans* (138). However, as a future experiment, it will be interesting to determine whether PASS2-3 produce less HCN compared PAO1. On the contrary, compared to PAO1, expression of cyanide-insensitive oxidase, CioA (PA3930) was elevated in PASS2 and PASS3. Both CioA and B are known to be induced at the stationary phase of growth or under hypoxic conditions (120). Although, in the present study PASS strains were grown under ambient oxygen supply, it is likely that PASS strains may have elevated baseline expression of CioA compared to PAO1, which could help the bacteria for adaptation under cyanogenic environment, typical of CF lungs.

Another interesting observation made in this study was altered expression of proteins involved in PQS biosynthesis in PASS strains, compared to PAO1. PQS was measured in bronchoalveolar lavage fluid, sputum and mucopurulent fluid of CF lungs, during lung transplantation, indicating that PQS is important for *P. aeruginosa* cell-cell signalling *in vivo* during chronic CF colonisation (192). The majority of PQS biosynthesis proteins were increased in abundance in PASS4 and decreased in abundance in PASS2-3 and unchanged in PASS1, compared to PAO1. The differential abundance might be one of the likely contributing factors for increased and decreased cytotoxicity of PASS4 and PASS2-3 respectively (138). This is consistent

with the previous observation by Gallagher *et al* that, PQS is essential for *P. aeruginosa* virulence in a *C. elegans* killing assay (191).

Virulence, can be defined as the ability of a pathogen to cause disease (201). In the CF lung the availability of resources is limited, and any selective advantage enjoyed by the virulence factor producing strain, would perhaps come at a cost of an elevated metabolic rate. Hence, variation in the production of virulence factors in the PASS strains suggests that the bacterium is choosing the virulence factor as per the need of the situation and it also gives a message that the *P. aeruginosa* virulence is multifactorial in nature.

One of the confounding observations made in this study was down-regulation (ranging ~1.5-3.8 fold) of the majority of the proteins in relation to aerobic respiration in PASS strains compared to PAO1, when grown aerobically in LB broth. Additionally, few but not all detected denitrification enzymes involved in anaerobic respiration of *P. aeruginosa* were differentially expressed heterogeneously in PASS strains compared to PAO1. This may indicate a lowered rate of metabolism in PASS strains as an adaptive strategy to the limited availability of oxygen in the stagnant mucus plugs of CF lungs compared to PAO1. However, there was no significantly apparent decrease in growth rate of PASS strains in comparison to PAO1 when grown planktonically in nutrient rich LB broth with an ambient supply of oxygen. Interestingly, majority of *cbb₃* oxidase sub-units were down-regulated in PASS strains compared to PAO1 when grown in aerobically. Previous studies have demonstrated *cbb₃* oxidases, specifically *Cbb₃*-2 is expressed under hypoxic condition (128, 178). Hence as a future experiment it will be interesting to compare expression of *Cbb₃* oxidases between PASS strains and PAO1 on exposure to hypoxic stress. Considering PASS strains have been isolated from CF lungs, it is probable that they may respond better to hypoxic stress via modulation of expression of the proteins including *cbb₃* oxidase, among many others.

Additionally, the presumed explanation for down-regulation of house-keeping protein in PASS strains compared to PAO1, including dehydrogenases and ATPases

could be due to moon-lighting function of these proteins. The moonlighting function refers to the ability of a protein or peptide to have more than one biological action (202). In other bacteria, such moonlighting proteins have been implicated to act as a virulence factors and also known to illicit immune response (202-206), which is an undesirable trait for *P. seruginosa* including PASS strains, dwelling in CF host's lungs. However, there have been relatively few studies on *P. aeruginosa* protein moonligthing (207-209) and hence, the field warrants more investigation, perhaps using clinical isolates.

4.5 Limitation of the study:

Luria-Bertani broth medium, utilised for growing PASS and PAO1 strains in this study, although rich in nutrients, may not fully represent the micro-nutrient availability in CF patient sputum (Section-1.4.6) (141). Hence, it will be worthwhile to compare the proteomes of CF isolates with PAO1 following growth in defined media such as SCFM.

4.6 Conclusions:

In the current study, we observed unique but heterogeneous expression of several proteins associated with virulence, respiration and quorum sensing in PASS strains which were different compared to non-CF, laboratory strain PAO1. It is likely that such modulations may provide a selective advantage to the bacterium for adaptation in a polymicrobial population in CF lungs. These results underscore *P. aeruginosa* strains adapted to the human host, specifically cystic fibrosis lungs have a unique set of adaptions which were also reflected in phenotype assays (138). Although much still needs to be understood, our work provides a context for future research to elucidate the potential role of the identified proteins as targets for therapeutic antagonists.

Chapter-5

Pseudomonas aeruginosa cell membrane protein expression from phenotypically diverse cystic fibrosis isolates demonstrate host specific adaptations

Rationale:

This chapter contains a published original research article;

Pseudomonas aeruginosa cell membrane protein expression from phenotypically diverse cystic fibrosis isolates demonstrate host-specific adaptations. Karthik Shantharam Kamath, Dana Pascovici, Anahit Penesyan, Apurv Goel, Vignesh Venkatakrishnan, Ian T Paulsen, Nicolle H Packer and Mark P Molloy. *J Proteome Res*. 2016 Jun 9.

In this chapter an iTRAQ-MS based quantitative comparison of enriched membrane sub-proteome of *P. aeruginosa* PASS1-3 strains with PAO1 was performed when grown in lung nutrient mimicking condition (SCFM) and a laboratory medium M9-glucose minimal medium. Comparison of membrane proteome profiles of PASS1-3 strains with non-CF strain PAO1 enabled to gain insights into CF specific modulations in the cell membrane. The proteomic findings were validated using functional assays including antibiotic MIC assay, motility and adhesion assays.

Contributions:

Bacterial cell culture, membrane protein extraction, proteolytic digestion, iTRAQ labelling mass spectrometric analysis, functional assays and data interpretation were performed by me. Statistical analysis was performed by Dr. Dana Pascovici. Sputum binding assay were permed by me and Dr. Vignesh Venkatakrishnan. The entire manuscript was written by me and read and approved by Prof. Mark Molloy.

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Pseudomonas aeruginosa Cell Membrane Protein Expression from Phenotypically Diverse Cystic Fibrosis Isolates Demonstrates Host-Specific Adaptations

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Ⓢ Supporting Information

ABSTRACT: *Pseudomonas aeruginosa* is a Gram-negative, nosocomial, highly adaptable opportunistic pathogen especially prevalent in immuno-compromised cystic fibrosis (CF) patients. The bacterial cell surface proteins are important contributors to virulence, yet the membrane subproteomes of phenotypically diverse *P. aeruginosa* strains are poorly characterized. We carried out mass spectrometry (MS)-based proteome analysis of the membrane proteins of three novel *P. aeruginosa* strains isolated from the sputum of CF patients and compared protein expression to the widely used laboratory strain, PAO1. Microbes were grown in planktonic growth condition using minimal M9 media, and a defined synthetic lung nutrient mimicking medium (SCFM) limited passaging. Two-dimensional LC-MS/MS using iTRAQ labeling enabled quantitative comparisons among 3171 and 2442 proteins from the minimal M9 medium and in the SCFM, respectively. The CF isolates showed marked differences in membrane protein expression in comparison with PAO1 including up-regulation of drug resistance proteins (MexY, MexB, MexC) and down-regulation of chemotaxis and aerotaxis proteins (PA1561, PctA, PctB) and motility and adhesion proteins (FliK, FlgE, FliD, PilJ). Phenotypic analysis using adhesion, motility, and drug susceptibility assays confirmed the proteomics findings. These results provide evidence of host-specific microevolution of *P. aeruginosa* in the CF lung and shed light on the adaptation strategies used by CF pathogens.

KEYWORDS: *Pseudomonas aeruginosa, membrane proteome, mass spectrometry, proteomics, virulence, bacterial evolution and adaptation*
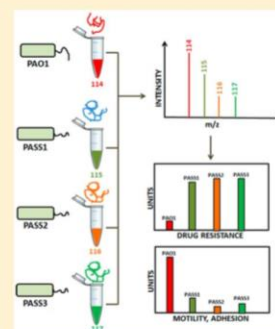
■ INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative, ubiquitous, opportunistic pathogen with a versatile genome (∼5500 genes) that provides capacity to adapt and thrive under diverse conditions.[1,2] Multidrug-resistant, biofilm-forming, and hyper-mutable strains of *P. aeruginosa* are common etiological agents under conditions including burn wounds, immunocompromised conditions (e.g., HIV), and cystic fibrosis (CF). CF is a recessive, congenital disease caused by a mutation in the cystic fibrosis conductance regulator (CFTR) gene. CFTR mutation leads to impaired movement of electrolytes across the cell, resulting in the formation of thick mucus, which, in turn, creates a favorable environment for the establishment and growth of microbial pathogens. One of the major causes of CF-related morbidity and mortality is infection by *P. aeruginosa*. Studies show that *P. aeruginosa* infection accounts for 60–70% of all respiratory tract infections in CF patients by the age of 20.[3] Despite host inflammatory defense responses and antibiotic treatment, *P. aeruginosa* causes chronic infections that persist for the lifetime of patients.

Chronic *P. aeruginosa* infections are associated with adaptation in highly compartmentalized, heterogeneous micro-environments of CF patient lungs.[3] It is likely that genetic

flexibility of *P. aeruginosa* residing inside lung mucus plugs enables the pathogen to specialize in metabolism as per the availability of surrounding nutrients.[4] Growth under these conditions also requires combating neighboring bacterial and fungal pathogens and host defenses. *P. aeruginosa* achieves this by expression of an array of surface-associated and secreted virulence factors including lipopolysaccharides, phenazines, hydrogen cyanide (HCN), exoenzyme S, and proteases (LasA, LasB).[5] Available nutrients are an important trigger to induce expression of many of these factors. Therefore, it is important to consider bacterial culture conditions in studies aimed at investigating growth in microenvironmental conditions. Proteins of bacterial cell envelope (outer membrane, periplasm, cytoplasmic membrane) often represent the first point of contact with the environment and play pivotal roles in establishing virulence, host cell adhesion, acquiring nutrients, drug efflux, signal transduction, quorum sensing (QS), and escaping host immune system responses. Despite the important contributions of cell envelope proteins in establishing and maintaining virulence in CF, there are almost no studies

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focused on characterization of these proteins in *P. aeruginosa* strains isolated from CF patients, with the majority of our knowledge derived from studies of the cell envelope of the burn wound isolate strain, PAO1.[6−10]

We recently reported whole genome sequences and global proteomic characterization from four CF isolate strains of *P. aeruginosa* grown in LB medium.[11] This showed that CF strains had similar sized genomes (ranging from 6.1 to 6.4 Mbp) and shared common core features, many of which were different from PAO1. These genomic features of CF strains were reflected in phenotypes including altered biofilm formation, pigmentation, and virulence. Global proteomic analysis revealed ~50% unique proteins were shared between CF strains compared with PAO1. CF strains expressed proteins involved in the biosynthesis of several nutrients, whereas these proteins were not required by PAO1 grown in LB medium. These results suggested the genetic flexibility of CF strains contribute toward survival and adaptation in CF lungs.

To better understand the roles of membrane proteins contributing to the adaptability of *P. aeruginosa* in the CF lung, we cultured three clinical isolates and PAO1 in a defined synthetic CF medium (SCFM) that mimics nutrient conditions in the CF lung[12] and compared membrane proteomes. We contrasted these conditions with growth in the M9-glucose minimal medium through the use of 2-D liquid-chromatography-coupled mass spectrometry (MS) of 4-plex iTRAQ-tagged peptides.[13] We validated the proteomic results using various functional assays to demonstrate the importance of drug-resistance proteins, chemotaxis, and adhesion in contributing to the specialization of *P. aeruginosa* adaptation in CF lungs.

■ EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

P. aeruginosa strains PASS1, PASS2, PASS3,[11] and PAO1 (ATCC 15692) (Supplementary Table S1) were grown on solid Luria−Bertani (LB) agar. For proteomic experiments bacteria were inoculated and grown in the synthetic cystic fibrosis medium (SCFM)[12] and M9 minimal salts-glucose (M9) medium directly from the frozen stock solution with minimal passaging. SCFM medium was prepared as per Palmer et al.[12] with modification. In brief, all amino acid stock solutions were prepared in 100 mM concentration in deionized water except for tryptophan, aspartate, and tyrosine, which were resuspended in 0.2, 0.5 M, and 1.0 M sodium hydroxide, respectively. For the final preparation of SCFM, amino acid stocks were added to a buffered base solution per liter of SCFM: 6.5 mL of 0.2 M NaH_2PO_4, 6.25 mL of 0.2 M Na_2HPO_4, 0.348 mL of 1 M KNO_3, 0.122 g NH_4Cl, 1.114 g KCl, 3.03 g NaCl, 10 mM MOPS, and 779.6 mL of deionized water. Volumes of the amino acid stock solutions added are described in Supplementary Table S2. The pH was adjusted to 6.8 and the medium was filter-sterilized. Sterile contents were added to the medium (per liter): 0.606 mL of 1 M $MgCl_2$, 1.754 mL of 1 M $CaCl_2$, and 1 mL of 3.6 mM $FeSO_4·7H_2O$. The M9-glucose medium was prepared as per vendor's instructions (Sigma, USA).Overnight bacterial cultures were diluted 1:100 using respective medium and grown in biological triplicates ($n = 3$) at 37 °C and constant shaking at 200 rpm until mid-logarithmic phase (Supplementary Figure S1). Cell pellets were collected by centrifugation at 2500g for 10 min at 4

°C and washed thrice with phosphate-buffered saline (PBS), pH 7.4.

Cell Lysis and Membrane Protein Enrichment

Cell pellets were resuspended in 0.5 mL of PBS containing benzonase (1:100 v/v, Sigma, USA), cOmplete EDTA-free protease inhibitor cocktail tablet (Roche, Germany), and an equal amount of acid-washed glass beads (Sigma, USA). Cells were lysed by bead-beating using a FastPrep FP120 bead-beater apparatus (Savant, USA). After lysis, centrifugation at 2500g for 8 min at 4 °C was performed to remove cell debris. Membrane proteins were enriched as previously described.[14] In brief, cell lysates were treated with ice-cold 100 mM sodium carbonate solution for 1 h at 4 °C, followed by ultracentrifugation at 115 000g at 4 °C. The pellet was washed twice with PBS, followed by another round of ultracentrifugation. The resultant membrane pellet was resuspended in 1% SDS in water (w/v). The sample was cleaned by acetone precipitation and resuspended in 250 mM triethylammonium bicarbonate (TEAB) with 0.05% SDS. Protein concentration was determined using Direct Detect (Merck Millipore, USA) according to the manufacturer's instructions.

In-Solution Digestion, iTRAQ Labeling, and Mass Spectrometry (MS)

For in-solution digestion, 60 μg of protein from each sample was reduced with 5 mM Tris(2-carboxyethyl) phosphine (TCEP) at 60 °C for 1 h and alkylated with 10 mM methylmethane thiosulfonate (MMTS) at room temperature for 10 min, followed by digestion with trypsin (Promega, USA) in 1:20 ratio (trypsin/protein) at 37 °C overnight. Digested proteins were lyophilized and resuspended in 0.5 M TEAB and labeled with iTRAQ 4-plex reagent as per manufacturer's instructions (Applied Biosystems, USA). Labeling efficiency was checked by MALDI-TOF/TOF. Samples were pooled at equal ratios then dried by vacuum centrifugation. Samples were cleaned using Sep-Pak Light C18 cartridge (Waters, USA) and separated into 15 fractions by strong cation exchange (SCX) chromatography, as described in Schilter et al.[15] Fractions were dried and resuspended in 0.1% TFA, 2% acetonitrile. Samples were analyzed using nano-LC−MS/MS coupled to a TripleTOF 5600 mass spectrometer (ABSciex, USA) with positive nanoflow electrospray analysis and information-dependent acquisition (IDA) mode. In IDA, MS/MS acquisitions of the 20 most intense m/z values exceeding a threshold >150 counts per second (cps) with charge states between 2+ to 4+ were selected for MS/MS analysis following a full MS survey scan and excluded for 20 s to minimize redundant precursor sampling.

Protein Identification and Quantitation

Protein identification and quantification was performed using ProteinPilot v4.2 software using the Paragon algorithm (ABSciex), thorough ID mode including biological modifications, and iTRAQ 4plex quantification. MS/MS data were searched against the database generated by combining the PAO1 protein sequence database retrieved from GenBank (release; December 2012) and in-house-generated in silico translated genome databases of PASS1−3 strains[11] (23 009 entries) with following settings: Cys alkylation: MMTS; digestion: trypsin; instrument: 5600, quantitative (iTRAQ-4 plex) and bias correction. An UnUsed Protein Score cutoff was set to 2.0 (99% protein identification confidence) and the FDR analysis using PSPEP algorithm was enabled. Peptides were

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quantified on the basis of the intensity of iTRAQ reporter ions via the Paragon algorithm after background correction and bias correction. Proteins were grouped using the ProGroup[16] algorithm included in the ProteinPilot program. Quantitation was filled down for competitors in each protein group[17] to obtain a realistic overlap between the protein identifications on different iTRAQ runs in the presence of a redundant search database.

Identification of Differentially Expressed Proteins

Protein quantities were combined from the three experiments, and differentially expressed proteins between the various strains and control were identified. The criterion used required the Stouffer combined p value to be <0.05 and consistent trends of differential expression across the triplicates as previously described by us.[18] Additionally, fold changes were required to be at least 1.3 fold, proteins had to be quantitated in at least two iTRAQ runs, and the trend of the quantification had to be similar across replicates. For the generation of GO annotation information for the proteins originating from PASS1−3 strains, the corresponding match in PAO1 locus tag (derived using an in-house nucleotide BLAST) was used. GO annotations were manually retrieved from PseudoCAP database (www.pseudomonas.com)[19] and assembled using Perseus 1.5[20] PloGO[21] and WeGO.[22] COG categories for proteins were derived from NCBI *P. aeruginosa* genome information.[23]

Bioinformatics Analysis and Predictions

For the prediction of the presence of transmembrane helices (TMH), we utilized TMHMM 2.0 server.[24] The N-terminal signal peptide was predicted using SignalP.[25] Grand average of hydropathicity (GRAVY) score was calculated (http://www.gravy-calculator.de/). Prediction of the subcellular location was performed using SOSUI-GramN.[26] Hypothetical proteins with no functional annotation were considered as potential membrane proteins if they contained at least one predicted TMH and SOSUI-GramN subcellular location prediction annotated as membrane. PAO1 GO annotation information in PseudoCAP database[19] was used for assignment of proteins into functional categories. Information in VFDB was utilized to assign proteins as virulence factors.[27] Information on *P. aeruginosa* PAO1 membrane transporters was manually retrieved from TransportDB database.[28]

Bacterial Sputum Binding Assay

The sputum sample was collected by noninvasive expectoration from a male patient aged 23 years and diagnosed with CF (ΔF508/ΔF508 mutation in CFTR gene) at Westmead Hospital, Sydney. Human research ethics approval was obtained from the Sydney West Area Health service (HREC/10/WMEAD/180). The patient's sputum tested positive for the presence of mucoid and nonmucoid *P. aeruginosa*, and this was confirmed by plate assay.[29]The sample was processed as per the protocol detailed in Venkatakrishnan et al.[29] In brief, the sputum sample was reduced and alkylated with 10 mM DTT and 25 mM iodoacetamide, respectively. Intact cells, cell debris, and insoluble mucins were removed by centrifugation for 30 min at 10 000 rpm. Protein concentration was determined using Direct Detect Spectrometer (Merck Millipore, USA). AcroWell 96-well plates with BioTrace PVDF membrane (Pall Corporation, USA) were activated and washed with methanol and PBS, respectively, followed by saturation of the membrane with pretreated sputum. Unbound sputum was decanted, and the membrane was washed thrice with PBS.

Corresponding flagella (*fliC, fliD, flgE, flgK, flgL,* and *fliF* (encoding MS ring protein)) and pili (*pilJ*) gene transposon mutants of *P. aeruginosa* PA14 strain[30] along with PAO1 and PASS1−3 strains were used for binding assays. PA14, PAO1, and CF strains PASS1−3 were cultured out of frozen stock on LB agar plates with minimal passaging. Individual colonies of PA14 mutants were inoculated into LB broth and grown overnight. PASS strains and PAO1 were inoculated into SCFM medium and grown until mid log phase. Both PA14 and PASS1−3 strains were treated independently, and cells were harvested by centrifugation and washed twice with PBS, labeled with SYBR Green dye. Cell density was adjusted based on the OD_{600} of cultures. An equal number of labeled cells was added to the pretreated membrane in triplicate and incubated for 1 h with constant shaking at 300 rpm. Unbound cells were removed by washing with PBS thrice. Fluorescence intensity was measured at 458 (excitation) and 520 nm (emission) and normalized to background fluorescence of the bacterium and sputum alone. Fluorescence intensity was used as a measure of the binding capacity of the respective strain to the sputum.

Plate Motility Assay

PASS1−3 strains were inoculated in SCFM and grown overnight. The cells were collected by centrifugation and resuspended in SCFM, and density was normalized based on OD_{600}. An equal volume of each density-adjusted cell suspension was spot-plated on SCFM (0.4% agarose) semisolid plates, in biological triplicate, and incubated at 37 °C for 12 h. Plates were imaged and sizes of the colonies were used as the measure of motility.

Determination of Minimum Inhibitory Concentrations for Antibiotics

Minimum inhibitory concentration (MIC) of a panel of antibiotics for PASS1−3 strains and PAO1 was determined. We utilized three antibiotics (moxifloxacin, polymyxin, and tobramycin) from a selection of antibiotics used in CF patient treatment (Supplementary Table 1). Colonies of PASS1−3 and PAO1 strains were inoculated and grown in an overnight culture in SCFM until the mid-log phase. Cell suspension turbidity was adjusted to $OD_{600} = 0.7$. An equal number of cells were inoculated into 96-well plates containing serially diluted antibiotics prepared in SCFM in triplicate. A drug-free SCFM medium blank control was also included. Plates were incubated on an orbital shaker at 37 °C until the mid log phase. Growth was measured at 600 nm and compared with the blank control.

■ RESULTS AND DISCUSSION

Experimental Overview

The aim of this work was to characterize the membrane subproteomes of *P. aeruginosa* strains isolated from sputum of CF patients grown in SCFM and M9-glucose medium. We used iTRAQ-MS to compare the membrane protein profiles of three clinical CF strains of *P. aeruginosa* (PASS1, PASS2, and PASS3)[11] to the well-characterized laboratory/reference strain, PAO1 (Supplementary Table 1). Previously, Palmer et al.[12] demonstrated that the growth rates, nutritional preferences, and gene expression profiles of *P. aeruginosa* when grown in SCFM and CF patient's sputum are similar. Hence, we rationalized that growth in SCFM would mimic the nutritional conditions of CF lungs and induce expression of different membrane proteins compared with M9-glucose minimal medium. Because the strains used in this study were obtained from CF patients of

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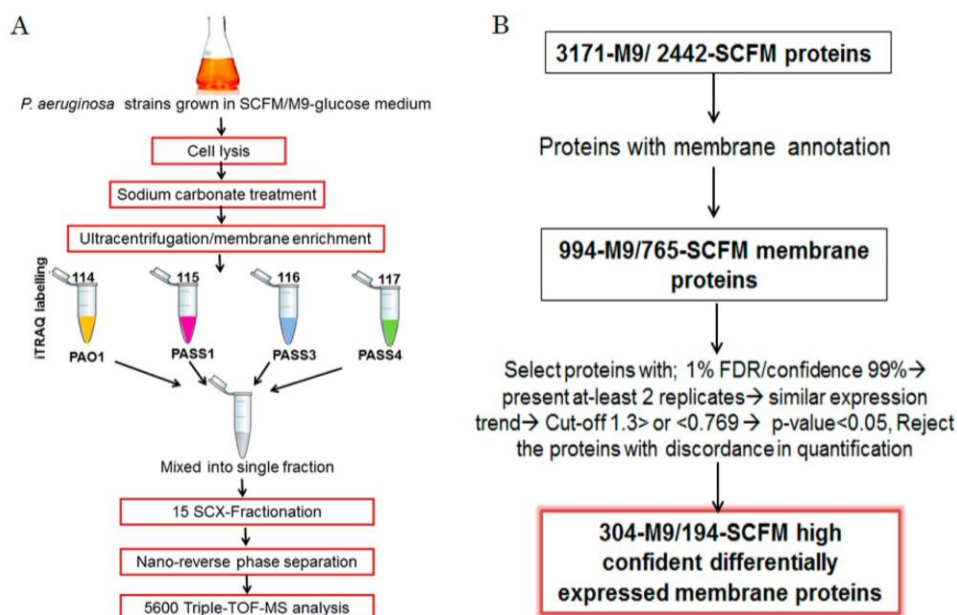
Figure 1. Workflow and data analysis pipeline. (A) Sample analysis workflow for 2D-LC-iTRAQ-MS/MS analysis of membrane enriched subproteome of *P. aeruginosa* grown in SCFM and M9-glucose medium. (B) Data analysis schema.

different age groups, disease severity, and exposure to antibiotic medications (Supplementary Table 1), they illustrate some of the diverse genotypic and phenotypic characteristics of *P. aeruginosa* observed clinically. Our study is unique because all previously reported *P. aeruginosa* membrane proteome studies[6−10] used model strains such as the wound isolate PAO1, which does not necessarily represent the genotypes and conditions needed for adaptation to the CF lungs.[31]

The experimental design used in this study is outlined in Figure 1A,B. Bacterial cells were collected at mid-logarithmic growth (Supplementary Figure S1), lysed, then enriched for membrane proteins after treating with sodium carbonate prior to ultracentrifugation.[14] We utilized 2D-LC/MS-MS coupled to iTRAQ 4-plex labeling for comparative quantitation. Six independent experiments representing three biological replicates of cells grown in the two different media were performed.

Bioinformatics Characterization of Identified Proteins

Mass spectrometry profiling of *P. aeruginosa* strains revealed 3171 and 2442 proteins (Global protein FDR < 0.06% and FDR < 0.08% for M9 and SCFM, respectively) in M9 and SCFM growth media, respectively (Figure 2A, Supplementary Figure S2, Table 1A,B, and Supplementary Table S3). This represents 46 and 36% of the proteome encoded in the PAO1 genome.

We next classified the proteins according to gene ontology information available in PseudoCAP database to determine protein localization (Figure 3). In cases where the GO localization category was "unknown", we used THMHH and SOSUI-GramN predictions that provided annotation of an additional 190 and 109 membrane proteins from M9 and SCFM, respectively (Supplementary Table S3). As an example, we detected a protein from the PASS2 strain, "Beta-propeller domains of methanol dehydrogenase type" (ID: NO-DE_114_l_112418_c_20.313990_45, Supplementary Table S3), which did not have an ortholog in PAO1; however, manual bioinformatics analysis predicted 16 TMH and 99.4%
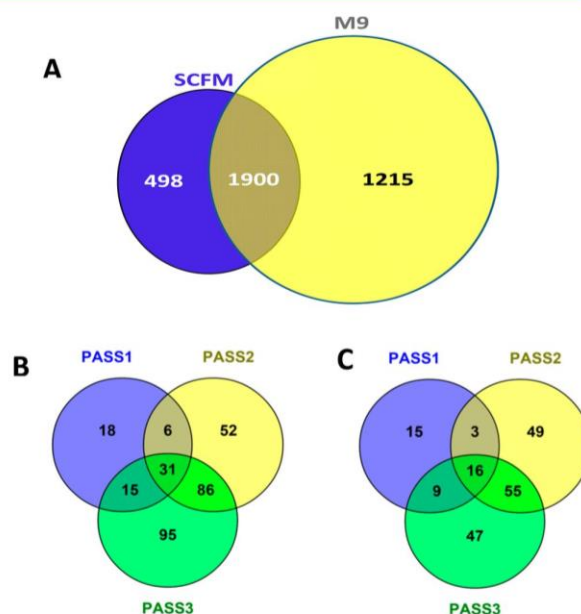
Figure 2. Overlap of identified proteins from M9-Glucose and SCFM growth conditions (A). Overlap of identification of differentially expressed proteins between PASS strains in M9-glucose (B) and SCFM medium (C).

sequence similarity with a "hypothetical protein" (PseudoCAP accession: 1695053, predicted location: cytoplasmic membrane) from the multi-drug-resistant *P. aeruginosa* NCGM2.S1 strain. Prediction of transmembrane helices (TMHs) using the bioinformatics tool THMHH (http://www.cbs.dtu.dk/services/TMHMM/) revealed 827 and 579 proteins with at least one TMH in M9 and SCFM cultures, respectively (Figure 4A). 87 and 42 proteins were predicted with >10 TMH in M9

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Table 1. (A) Summary of *P. aeruginosa* Protein Identification Using 2D-LC-iTRAQ-MS/MS Analysis and (B) Summary of Differentially Expressed Proteins Identified in All the Strains In SCFM and M9-Glucose Growth Condition

A			
media of growth	unused score (confidence) cutoff	proteins detected	calculated global protein FDR
M9-glucose	>2(99%)	3171	0.0006
SCFM	>2(99%)	2442	0.0008

B		
ratio	differentially expressed proteins M9-Glucose[a]	differentially expressed proteins SCFM[a]
115:114 (PASS01/PAO1)	45 (15 up, 30 down)	43 (22 up, 21 down)
116:114 (PASS02/PAO1)	175 (88 up, 87 down)	123(54 up, 69 down)
117:114 (PASS03/PAO1)	225 (67 up, 158 down)	127 (33 up, 94 down)

[a]Cutoff criterion: Protein confidence 99%→ present in at-least two replicates→ similar expression trend→ Cutoff >1.3 or <0.769 → p value < 0.05.
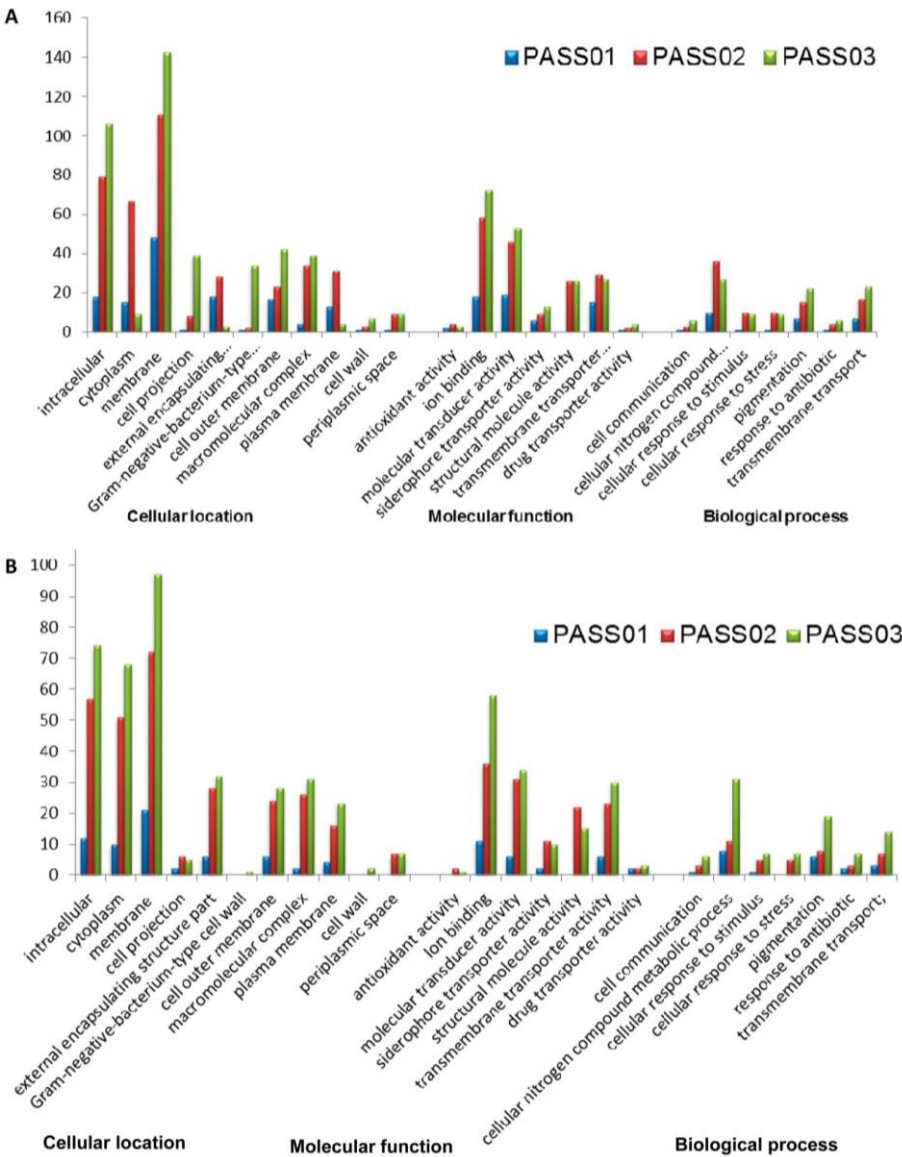
Figure 3. Functional GO category classification of all the identified proteins. Comparison of GO categories of proteins identified across the strains in (A) M9 medium and (B) SCFM medium.

and SCFM cultures, respectively (Figure 4A). Grand average hydropathy (GRAVY) score was used to document the hydrophobicity of the identified proteins, with positive GRAVY values representative of greater hydrophobicity.[32]

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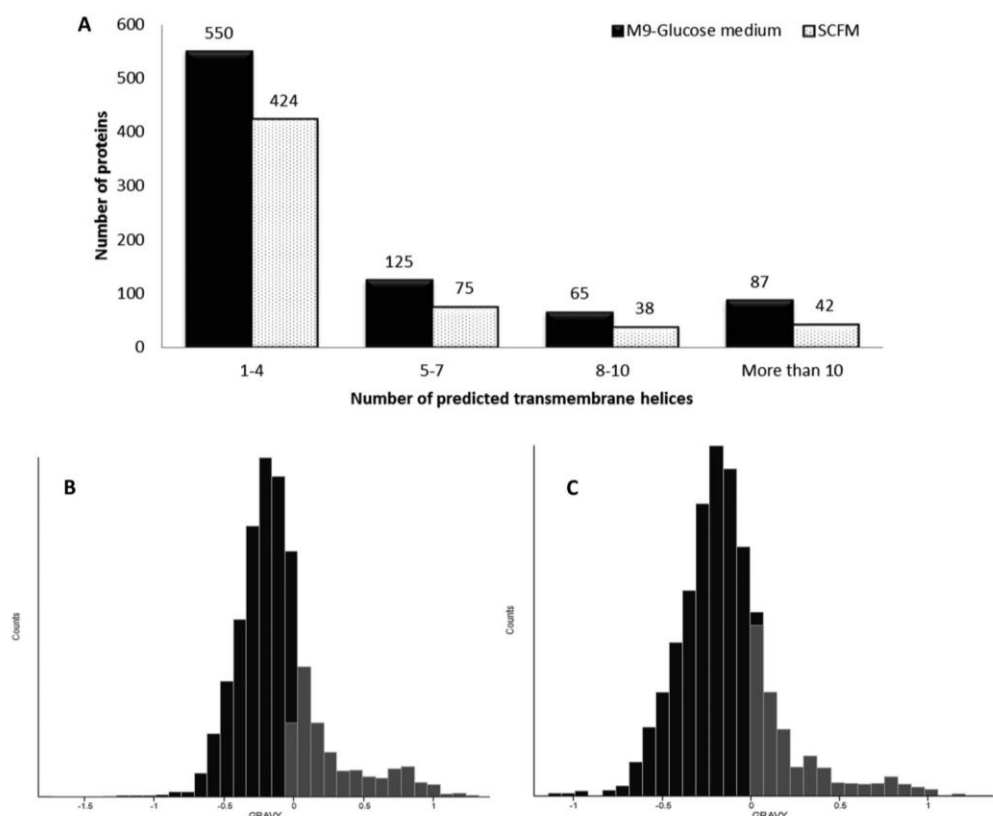
Figure 4. Bioinformatic predictions of membrane protein physicochemical properties. (A) Predicted transmembrane helices in M9 and SCFM growth conditions. Distribution of grand average of hydropathy (GRAVY) scores for P. aeruginosa proteins identified from (B) M9-glucose medium and (C) SCFM medium. Gray represents proteins with positive GRAVY values.

800 and 557 proteins had positive GRAVY values in M9 and SCFM, respectively (Figure 4B,C).

Secretory proteins in Gram-negative microbes shuttle through the periplasmic space before they are released and can be identified by the presence of an N-terminal signal peptide.[25] Hence, membrane protein enrichment often contains secretory proteins. We used the SignalP tool[25] that predicted the presence of signal peptide cleavage sites in 433 and 350 proteins in M9 and SCFM media, respectively. In summary, the bioinformatics assignments and predictions yielded annotation for 994 (31%) and 765 (31%) nonredundant membrane proteins from M9 and SCFM cultures, respectively (Supplementary Table S3). More than 10 years ago, a global membrane proteome profiling study of *P. aeruginosa* PAO1 grown in LB reported 20% of identified proteins as membrane proteins, and no quantitative information was reported.[7] Thus, the present study adds substantially to our knowledge of membrane protein expression in clinically relevant *P. aeruginosa* strains.

Mass Spectrometry Comparison of the Membrane Proteomes of CF Strains and PAO1

Proteins present in at least two of three replicates with significant fold change ratio versus PAO1 and with similar trends of expression across the replicates were considered to be differentially expressed using criteria we previously reported for iTRAQ analysis.[17,18] Because of high sequence similarity across the different strains for some proteins, peptides may be shared, which complicates detecting quantitative changes. For instance, protein PilA of PAO1 has 81% sequence similarity with that of

PASS1 and PASS3 and 40% with PASS2 (Supplementary Figure S3).

In such a situation, it is possible to have PilA (PAO1) appear up-regulated and PilA (PASS3) down-regulated, leading to confusing results. In the current report, we label such conflicts as "identified but not quantified" and not considered in the list of differentially expressed proteins. As a result, of this stringent filtering, we present a high confident quantification of 304 and 194 membrane proteins from M9 and SCFM growth conditions, respectively (Supplementary Table S4).

Using PAO1 expression as the reference, PASS1 displayed fewer differentially expressed proteins 45 and 43 in M9 and SCFM (Table 1B) compared with PASS2 and PASS3. This is consistent with the observations that PASS1 and PAO1 displayed more similar growth patterns (Supplementary Figure S1) and phenotypic characteristics including the production of phenazines and biofilm formation.[11] The PASS3 strain showed the greatest numbers of differentially expressed proteins with 225 and 127 in M9 and SCFM, respectively. PASS2 and PASS3 shared many common differentially expressed proteins, 86 and 55 in M9-glucose and SCFM, respectively (Figure 2B,C), while the overlap in differentially expressed proteins across all PASS strains was small (31 and 16 proteins in M9 and SCFM, respectively). These findings reflect the varied genetic differences observed between clinical pathogen isolates.

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Table 2. Relative Abundance (*n*-fold change) of Representative Membrane Proteins of *P. aeruginosa* Strains PASS1−3 in Reference to PAO1 When Grown in M9-Glucose and SCFM Media[a]

A

PAO1 Locus tag	Uniprot Accession	Gene	Name	M9-Glucose						SCFM					
				PASS1/PAO1	P.value	PASS2/PAO1	P.value	PASS3/PAO1	P.value	PASS1/PAO1	P.value	PASS2/PAO1	P.value	PASS3/PAO1	P.value
PA0180	Q9I6V2	cttP	Chemotactic transducer for trichloroethylene [positive chemotaxis], CttP	0.5938	0.0741	0.3445	0.0269	0.2602	0.1019						
PA0411	P42257	pilJ	twitching motility protein PilJ	0.4078	0.1050	0.4202	0.0570	0.2457	0.0000						
PA1082	Q9I4P7	flgG	flagellar basal body rod protein FlgG	0.3238	0.2939	0.3262	0.0418	0.2288	0.1243						
PA1086	Q9I4P3	flgK	flagellar hook-associated protein FlgK	0.5293	0.4173	0.2330	0.0018	0.2243	0.0033	0.5962	0.2661	0.2194	0.0446	0.1880	0.0951
PA1087	Q9I4P2	flgL	flagellar hook-associated protein FlgL	0.8402	0.2147	0.1607	0.0002	0.1352	0.0021	0.8445	0.5880	0.2481	0.0481	0.2421	0.0344
PA1094	Q9K3C5	fliD	flagellar capping protein FliD	0.2021	0.4155	0.1145	0.1230	0.1090	0.0293	0.2377	0.0213	0.1084	0.0228	0.1080	0.0159
PA1101	Q51463	fliF	flagellar MS-ring protein	0.6719	0.6704	0.2490	0.0064	0.2487	0.0097	0.4056	0.1563	0.0441	0.0025	0.0767	0.0013
PA1445	Q51467	fliO	flagellar protein FliO	0.9745	0.8201	0.5120	0.2189	0.4171	0.0164						
PA1454	G3XD64	fleN	flagellar synthesis regulator FleN							0.7886	0.9320	0.6312	0.0367	0.8484	0.0594
PA1460	G3XD73	motC	flagellar motor protein	1.7876	0.0391	0.4765	0.0066	0.5193	0.0045						
PA1461	G3XD90	motD	flagellar motor protein MotD	1.3959	0.6785	0.3418	0.0952	0.3479	0.0179						
PA1561	Q9I3F6	aer	aerotaxis receptor Aer	0.6090	0.1072	0.2127	0.0000	0.2190	0.0000	0.6789	0.2518	0.2452	0.0001	0.2475	0.0006
PA1608	Q9I3B3	-	chemotaxis transducer	0.5909	0.0032	0.5809	0.0006	1.2215	0.0082	0.7312	0.2647	2.2227	0.0055	0.8698	0.0884
PA2652	Q9I0I6	-	chemotaxis transducer	0.6582	0.2123	0.1682	0.0000	0.1638	0.0000	1.4042	0.8842	0.3743	0.0703	0.2832	0.0130
PA2654	Q9I0I4	-	chemotaxis transducer	0.5560	0.0493	0.1392	0.0000	0.1194	0.0000	0.6433	0.2131	0.1004	0.0002	0.1113	0.0002
PA2788	Q9I0S5	-	chemotaxis transducer	1.0162	0.6028	0.1704	0.0000	0.1879	0.0000						
PA2867	Q9HZX9	-	chemotaxis transducer	0.8473	0.2305	0.2336	0.0002	0.2169	0.0001						
PA2920	Q9HZS7	-	chemotaxis transducer							0.7272	0.4745	0.2682	0.0076	0.2833	0.0038
PA3708	Q9HXT3	wspA	chemotaxis transducer	0.9474	0.7643	0.8908	0.0026	0.4592	0.0001						
PA4290	Q9HWB0	-	chemotaxis transducer							1.3398	0.1584	0.3841	0.0036	0.9016	0.0111
PA4309	G3XD24	pctA	chemotactic transducer PctA	1.1628	0.9488	0.6029	0.0187	0.5083	0.0109	1.8867	0.0141	0.2703	0.0111	0.2137	0.0037
PA4310	Q9HW91	pctB	chemotactic transducer PctB	0.3410	0.2982	0.0981	0.0000	0.0902	0.0000	1.0344	0.3398	0.0742	0.0164	0.1199	0.0059
PA4520	Q9HVQ2	-	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)							0.3516	0.5820	0.1445	0.1949	0.1305	0.0351
PA4551	G3XD84	pilV	type 4 fimbrial biogenesis protein PilV	0.8955	0.7859	3.5892	0.0247	1.2178	0.5351						
PA4553	Q9HVM9	pilX	type 4 fimbrial biogenesis protein PilX	0.5390	0.0475	1.8633	0.0156	0.9460	0.2388						
PA4554	Q9HVM8	pilY1	type 4 fimbrial biogenesis protein PilY1	1.4251	0.9854	2.3816	0.0406	1.2830	0.6860						
PA4633	Q9HVF8	-	chemotaxis transducer	1.2556	0.6564	0.3431	0.0000	0.4274	0.0004	0.4555	0.0228	0.1764	0.0000	0.1839	0.0000
PA4953	Q9HUL2	motB	flagellar motor protein MotB	0.8900	0.7040	0.9080	0.0250	0.5915	0.0004	0.5828	0.5438	0.8156	0.0266	0.3731	0.0002
PA5040	P34750	pilQ	type 4 fimbrial biogenesis outer membrane protein PilQ precursor	0.6397	0.3496	0.6802	0.0487	0.2827	0.0000						
PA5041	G3XCX7	pilP	type 4 fimbrial biogenesis protein PilP	0.9896	0.8758	0.8694	0.0004	0.2415	0.0007						
PA5042	G3XD51	pilO	type 4 fimbrial biogenesis protein PilO	0.4786	0.3066	0.3798	0.0176	0.2607	0.0001						
PA5043	G3XD30	pilN	type 4 fimbrial biogenesis protein PilN	0.5078	0.0262	0.9101	0.0037	0.2895	0.0000	0.4319	0.0985	0.8384	0.2425	0.3608	0.0151
PA5072	Q9HUB1	-	chemotaxis transducer	0.2409	0.2752	0.1719	0.0107	0.1184	0.0046						

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PAO1 Locus tag	Uniprot Accession	Gene	Name	PASS1/PAO1	P.value	PASS2/PAO1	P.value	PASS3/PAO1	P.value	PASS1/PAO1	P.value	PASS2/PAO1	P.value	PASS3/PAO1	P.value
PA0158	Q9I6X4	-	Resistance-Nodulation-cell Division (RND) efflux transporter	1.8670	0.0456	0.8430	0.1912	0.6975	0.0119						
PA0426	P52002	mexB	Resistance-Nodulation-cell Division (RND) multidrug efflux transporter MexB							1.7979	0.0335	1.0558	0.0351	0.7385	0.0249
PA0427	Q51487	oprM	multidrug ABC transporter	0.7579	0.0874	1.2245	0.0008	1.5859	0.0000						
PA0807	Q9I5D1	ampDh3	AmpDh3	0.9612	0.7805	4.6184	0.0009	0.7186	0.5108						
PA1032	Q9I4U2	quiP	Penicillin acylase (EC 3.5.1.11)	0.7655	0.4063	0.8804	0.1753	0.4547	0.0000	1.0649	0.0026	1.4634	0.0182	0.8403	0.2990
PA1876	Q9I2M1	-	ABC transporter ATP-binding protein/permease	5.4216	0.0134	0.5918	0.0908	0.5014	0.3703						
PA2018	G3XCW2	-	multidrug efflux protein	2.0864	0.0000	3.5659	0.0000	4.5739	0.0000	4.4344	0.0205	2.4429	0.0063	2.2350	0.0001
PA2525	G3XCM8	-	hypothetical protein	0.8757	0.9737	1.4099	0.0007	1.9033	0.0325						
PA2528	Q9I0V5	-	Resistance-Nodulation-cell Division (RND) efflux membrane fusion protein	0.9834	0.6505	1.4012	0.0010	1.4760	0.0054						
PA2615	Q9I0M3	ftsK	DNA translocase FtsK	0.6876	0.0237	0.9195	0.7129	1.2056	0.9815	0.6131	0.0197	0.8729	0.5750	0.6283	0.0178
PA2760	Q9I083	-	hypothetical protein							3.6968	0.0217	5.3376	0.0000	3.2569	0.0000
PA3677	Q9HXW3	-	Resistance-Nodulation-cell Division (RND) efflux membrane fusion protein	1.8217	0.0633	0.5786	0.0708	0.4908	0.0186						
PA4597	Q51397	oprJ	RND efflux system, outer membrane lipoprotein, NodT family	1.1873	0.0340	1.8586	0.0000	4.8792	0.0000						
PA4599	G3XD25	mexC	Multidrug efflux RND membrane fusion protein MexC	1.1671	0.9659	3.1214	0.0517	10.5520	0.0064						
PA5159	Q9HU25	-	Membrane fusion component of tripartite multidrug resistance system	0.5929	0.8389	0.2617	0.0282	0.3434	0.0794						
PA5199	Q9HTZ0	amgS	protein AmgS	0.7877	0.2673	1.4712	0.0004	1.0632	0.0447						
PA5288	Q9HTR6	glnK	nitrogen regulatory protein P-II 2							1.0139	0.2166	1.6628	0.5378	2.0806	0.0165

[a]Membrane proteins detected in at least two biological replicates, with similar quantification trend and differential expression ratio >1.3 (shaded red; $p < 0.05$, shaded yellow) and <0.769. (shaded green; $p < 0.05$, shaded yellow) were considered to be statistically significantly altered. Locus tag refers to corresponding PAO1 locus tag. (A) List of representative proteins associated with motility, adhesion, chemotaxis, and aerotaxis of *P. aeruginosa*. (B) List of representative, differentially expressed proteins associated with drug resistance of P. aeruginosa.

Comparative Analysis of Membrane Protein Expression in SCFM and M9-Glucose Medium

In the CF lungs, *P. aeruginosa* frequently copes with limitation and variation in nutrient availability, which drives the evolution of tailored metabolism for survival advantage.[33] In our study, ~30% more proteins were detected from cells grown in M9-glucose minimal media (3171 proteins) compared with the growth in SCFM (2442 proteins). Interestingly, we observed differential expression of multiple virulence-associated proteins

in CF strains when grown in M9-glucose medium only (Supplementary Table S4).

For example, four proteins (AlgD, Alg44, AlgK, AlgE) involved in alginate biosynthesis were upregulated in CF strains compared with PAO1 when grown in M9 medium but not in SCFM. Several other proteins were differentially expressed when grown in SCFM but not in M9. For example, aminopeptidase (PA2939), OprQ porin involved in antibiotic resistance, outer membrane chaperon SurA (PA0594), and drug efflux proteins MexB (PA0426) were differentially

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expressed when grown in SCFM but not in the M9 medium. Metal acquisition proteins including copper (PA3790), iron/heme (PA2688, PA4710, PA0931), and magnesium (PA0913) transporters were seen to be differentially expressed under both the conditions, highlighting the importance of these cofactor metals in enabling *P. aeruginosa* CF strains to persist in the CF lung microenvironment.

CF Strains Have Decreased Motility and Adhesion Compared with PAO1

Bacterial motility is a key phenomenon essential for adhesion and colonization, ultimately leading to the formation of resistant biofilms. Motility in *P. aeruginosa* is enabled by flagella and pili. Both flagella and pili are known potent virulence factors as they provoke acute infection in the host. Hence, down-regulation of flagella expression is used as a "cloak" to escape both phagocytic receptor and TLR5-driven inflammation, thereby conferring a survival advantage.[34−37] Recently, Mayer-Hamblett et al.[38] using a large set of CF isolates of *P. aeruginosa* showed that pulmonary exacerbation correlated well with reduced twitching motility of the bacterium. We detected several *P. aeruginosa* motility and adhesion-related proteins of which 26 were differentially expressed and, interestingly, most of them were down-regulated in PASS1−3 compared with PAO1 in both growth media (Table 2A). For example, the abundance of FliD, FlgG, FliK, and FlgL was decreased in PASS2 and PASS3 in comparison with PAO1. Notably, sigma factor AlgU (PA0762), a predicted cytoplasmic protein, was detected in our analysis and was found upregulated in PASS2 and PASS3 when grown in M9 but not in SCFM. AlgU is known to negatively regulate flagellum biosynthesis by inhibiting expression of gene FleQ,[39] which is a positive regulator of adhesion.[40]

Flagella- and pili-based motility are guided by chemotaxis/aerotaxis, which is regulated by a two-component signaling system via ligand binding receptor modules that transduce the signal into a kinase-based pathway.[41] We observed down-regulation of several proteins involved in chemotaxis/aerotaxis in PASS1−3 under both growth conditions (Table 2A). For instance, aerotaxis receptor protein Aer (PA1561) was down-regulated in PASS2 and PASS3 in comparison with PAO1 under both growth conditions. Aerotaxis receptors sense the oxygen in the environment enabling the bacterium to migrate toward or away from it. Two methyl-accepting chemotaxis integral membrane proteins PctA and PctB were down-regulated in PASS2 and PASS3 strains. Both of these proteins are known to transduce signals across the membrane, thereby facilitating chemotaxis by varying the level of protein methylation. Overall, down-regulation of chemotaxis- and aerotaxis-associated regulators in comparison with PAO1 may indicate the limited necessity of motility inside the mucus plugs of CF lungs, which are often hypoxic and nutrient-limited and hence are not a good stimulus for chemotaxis or aerotaxis.

To functionally validate the iTRAQ data, we assessed the adhesion capacity of PASS strains by performing a CF sputum binding assay. The binding capacity of PASS1−3 strains to CF sputum was similar and 5-fold less than PAO1 (Figure 5A), consistent with the MS data. We extended this analysis by carrying out the binding assay using PA14 transposon insertion mutants of orthologous genes.[30] Selective transposon mutants of *fliD*, *flgE*, *flgK*, and *pilJ* genes showed reduced binding to CF sputum in comparison with PA14 wild-type (Figure 5B). Furthermore, nucleotide BLAST search of PASS strain
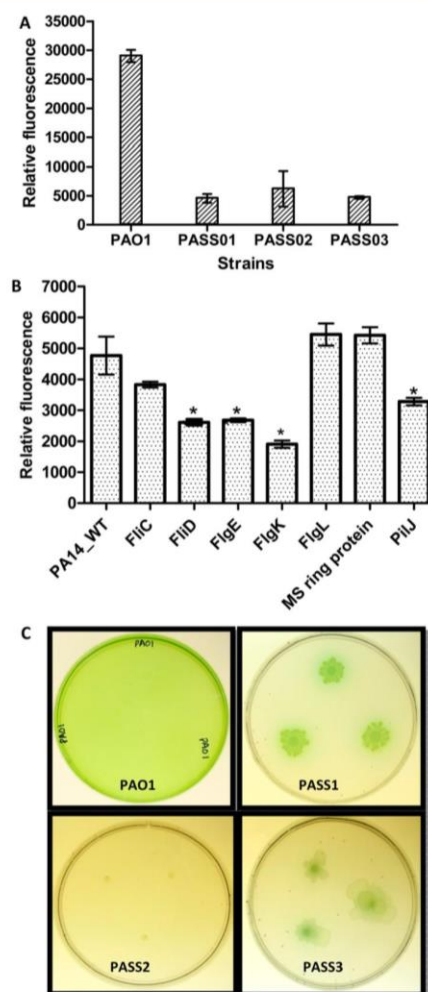
Figure 5. CF patient sputum binding assays. (A) PASS1−4 and PAO1 strains. (B) PA14 transposon mutants and wild-type strains. *$p < 0.05$ CF PA14 WT. (C) Plate motility assay of PASS1−3 and PAO1 ($n = 3$) on semisolid SCFM agarose (0.4%) plates.

genomes against PAO1 revealed the absence of the lectin gene (LecA) in PASS1−3 strains (data not shown), consistent with its absence in another CF-associated Australian epidemic strain, AES-1R. Lectins are demonstrated to be important in cell recognition and adhesion.[42] Hence, the absence of LecA gene in PASS1−3 is an additional factor lowering its adhesion capacity.

Although it is widely accepted that the downregulation of flagella helps *P. aeruginosa* to evade host immune system recognition, a recent study suggests that it is not the loss of flagellum per se, rather the loss of flagella-based motility that contributes to the evasion of phagocytic clearance.[36] Hence, we evaluated the motility of CF strains on SCFM, 0.4% semisolid agarose plates. We observed an overall reduced motility of CF strains compared with PAO1 (Figure 5C). PASS2 exhibited little or no flagella motility compared with PAO1, PASS1, and PASS3. The proteomics and functional data are consistent with CF strains showing reduced flagella expression. Hence, it is evident that the loss of motility provides a competitive advantage for *P. aeruginosa* strains in the CF patient lung.

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Elevated Expression of Drug-Resistance Proteins in CF Strains

As a result of clinical intervention, *P. aeruginosa* in CF patient lungs are continuously exposed to a variety of antimicrobial agents. We obtained the list of proteins categorized as "antibiotic resistance and susceptibility" from PseudoCAP database and TransportDB and matched these with experimentally identified ones. We identified 68 proteins collectively across all strains (Table 2B and Supplementary Table S3) that are known contributors in *P. aeruginosa* drug resistance, considerably higher than previous reports.[6−9] Furthermore, 19 membrane transporter proteins showed differential expression in PASS1−3 strains, in contrast with PAO1 mainly when grown in M9 medium compared with SCFM (Table 2B). This might be due to the need to improve transmembrane transport of nutrients due to lack of many of the common nutrients in M9 medium. An integral membrane multidrug efflux protein, MexY (PA2018), was found to be overexpressed in all PASS strains in comparison with PAO1 in both the media conditions (Supplementary Figure S4). MexY, MexX, and MexD identified in our study belong to the resistance−nodulation−division (RND) family of efflux proteins system, which functions as proton/drug antiporters widely found in Gram-negative bacteria and perform active efflux of an array of antibacterial agents.[43] Overexpression of MexY in CF strains in comparison with PAO1 has been previously reported, thereby demonstrating its vital role in drug resistance.[44] OstA "organic solvent tolerance protein" (PA0595) was detected under both M9 and SCFM growth conditions. The protein was upregulated when grown in SCFM in PASS2 and PASS3. OstA protein along with other efflux proteins has been extensively characterized in the Gram-negative bacterium, and it has been shown to confer tolerance to a wide range of organic solvents that would be otherwise toxic.[45] In *E. coli*, organic solvent-resistant strains were concomitantly shown to have elevated resistance to multiple antibiotics.[46]

To confirm the contribution of elevated levels of drug-resistance-associated proteins, we performed MICs using three antibiotics with diverse mechanisms of action from a selection of antibiotics used for the treatment of patients (Supplementary Table 1). All PASS strains showed increased resistance to the membrane targeting cyclic peptide antibiotic polymyxin and the fluoroquinolone DNA gyrase inhibitor drug moxifloxacin (Figure 6) in comparison with PAO1.

Among PASS strains, PASS2 showed highest resistance to moxifloxacin and MICs were twice that of PASS1 and PASS3. PASS2 and PASS3 showed similar MIC for polymyxin but
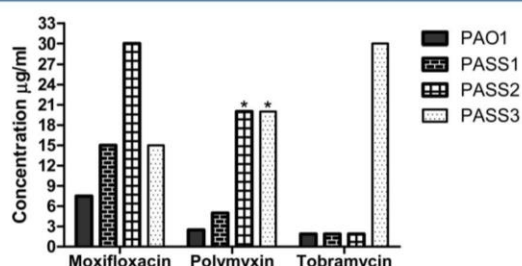

Figure 6. Antibiotic MIC assays of PASS1−3 and PAO1 grown in SCFM medium. Assays were performed in biological triplicates (*n* = 3). *Y* axis represents concentrations of antibiotic in *μ*g/mL. * Maximum concentration tested.

thrice that of PASS1. Moxifloxacin resistance of *P. aeruginosa* has been demonstrated by MIC assays in strains isolated from a large set of CF patients;[47] however, only PASS3 showed increased resistance to the aminoglycoside ribosome inhibitor tobramycin in comparison with PAO1. Although tobramycin is one of the most effective drug used to treat *P. aeruginosa* pulmonary infections, adaptive resistance to the drug has been previously reported by in vitro MIC assays performed using CF patient sputum.[48] One of the recent global proteome profilings of *P. aeruginosa* MPAO1 strain on exposure to subinhibitory concentrations of tobramycin reported up-regulation of a heat shock protein IbpA.[49] Notably, compared with PAO1, we observed over 2-fold increase in IbpA in PASS2 and PASS3 but not in PASS1, which is most similar to PAO1.

We noted that PASS1 isolated from a patient treated with combinations of ciprofloxacin, polymyxin, and tobramycin showed low in vitro MICs for polymyxin and tobramycin, suggesting that these compounds may be efficacious in this patient. Conversely, the PASS2 isolate showed high in vitro MIC to polymyxin, suggesting that prior in vivo exposure was linked with increased drug resistance. Furthermore, PASS3 isolated from a patient with exposure to the ribosome inhibitor clarithromycin showed high MIC to tobramycin, consistent with increased resistance to drugs targeting protein synthesis machinery. These results demonstrate that some CF strain isolates develop in vivo resistance to particular drug classes, but this was not uniformly demonstrated in the small number of isolates examined here.

CF Strains Portray Altered Expression of Virulence Factors

P. aeruginosa virulence factors are diverse including toxins, cell surface adhesion proteins, hydrolytic enzymes, and cell surface carbohydrates.[50] We extracted information on all of the known "membrane associated" virulence factors of the *P. aeruginosa* PAO1 from the Virulence Factors Database,[27] which reports 112 membrane-associated virulence factors (MAVFs). Of these, our MS experiment detected 75 MAVF in both growth media (Supplementary Table S3). Flagella- and pili-associated proteins represented a major class of MAVF detected in our study. We identified 16 flagella regulon proteins, of which 15 are known to be virulence factors. Flagellar filament structural protein FliC (PA1092) was one of the most abundant of all detected proteins in our MS analysis. We identified five pili-associated proteins (PA0410, PA3805, PA4525, PA4526, and PA4528) including PilA, which forms the monomer of pili. One of the pili-associated proteins detected in M9 medium, "arginine-specific autotransporter" (AaaA) of *P. aeruginosa* (PA0328), belongs to M28 family of peptidases with specific roles such as agglutination and vacuolating toxins and serine proteases. Luckett and colleagues recently demonstrated AaaA as a potential virulence factor and showed that lack of AaaA led to attenuation of the pathogenic bacterium in a mouse chronic wound infection, demonstrating its key role in the establishment of infection.[51]

Secretion systems are essential for the translocation of enzymes and other virulence factors across the membrane. Type I secretion system (T1SS) is involved in secretion of key alkaline proteases including AprX and HasAp, whereas T2SS plays a key role in the secretion of alkaline phosphatases, elastases (LasA, B), and hemolytic and nonhemolytic phospholipases and lipases.[52,53] Five proteins (HasD, AprX-AprD-AprE-AprF) of the T1SS apparatus and seven proteins (Xcp; Q, S, T, U, W, FimU) of theT2SS were identified in both

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M9 and SCFM media in all PASS strains. Of the T1SS-detected proteins, AprD, AprE, and AprF are membrane proteins that are necessary for secretion of alkaline protease (AprA), also known as aeruginolysin.[54] We observed downregulation of AprD and AprF in all three CF strains in comparison with PAO1 when grown in M9 medium but not in SCFM; however, AprA was observed to be downregulated in PASS2 and PASS3 but not in PASS1 in M9 medium. The downregulation of AprA and secretion apparatus might contribute to the reduced virulence of PASS2 and PASS3 strains. Recent work from our group demonstrated similar findings, where PASS strains demonstrated reduced virulence in comparison with PAO1 using a *C. elegans* grazing model.[11]

Alginate biosynthesis and conversion to a mucoid phenotype is a hallmark of CF-associated strains of *P. aeruginosa*, which have been linked with increased pulmonary exacerbations.[38] The conversion to a mucoid phenotype is often controlled by a set of sigma and antisigma factors residing in the periplasm and inner membrane. Out of 12 proteins involved in alginate biosynthesis,[55] we identified 10 (AlgD, Alg44, AlgK, AlgE, AlgG, AlgL, AlgI, AlgJ, AlgF, AlgA) in M9 and SCFM media collectively. Interestingly, most of the differential expression in comparison with PAO1 was observed in M9 growth condition. AlgD, Alg44, AlgK, and AlgE were upregulated mainly in PASS2 and PASS3 when grown in M9 condition but not in SCFM. A similar conclusion was drawn by Son et al.,[56] who used gene-expression studies to show that CF strains have significantly increased alginate biosynthesis compared with PAO1. Increased alginate production is known to lead to biofilm formation[57] thus providing a survival advantage to the bacterium inside the host. An overall trend of altered expression of virulence determinants in PASS strains suggests that this is an important survival strategy used in the host environment.

Identification of Porins

Porins are water-filled beta-barrel structured channels spanning the outer membrane of the Gram-negative bacterium, which acts as "molecular sieves" and are involved in the passive diffusion of hydrophilic molecules across the membrane. In recent years, they have gained considerable interest due to involvement in drug resistance.[58] We detected 17 porins, several substrate-specific porins including OprB and OprD, across all strains under both growth conditions. We observed up-regulation of the OprD protein in PASS1 but not in PASS2 and PASS3, in comparison with PAO1 when grown in both M9 and not in SCFM. OprD was one of the first characterized outer membrane proteins associated with beta-lactam resistance, where deletion variants were shown to exhibit increased susceptibility to multiple antibiotics.[58] OprF was downregulated in PASS2 and PASS3 in comparison with PAO1 when grown in SCFM. Recent studies demonstrated that isogenic OprF mutant lacked adhesion capacity, secretion of toxins, and production of QS-dependent virulence factors.[59] Reduced expression of virulence determinant porins might act as a contributing factor for reduced virulence of PASS2 and PASS3.

■ CONCLUSIONS

We quantitatively compared membrane subproteomes of three phenotypically diverse *P. aeruginosa* CF isolates grown in minimal media and SCFM and compared the expression levels to the widely studied reference strain PAO1. We observed considerable diversity in membrane proteome expression in CF

strains PASS1−3 in comparison with PAO1. PASS1 showed the most similar growth characteristics to PAO1, and this was reflected by the least differences in membrane protein expression compared with PASS2 and PASS3 strains. PASS2 and PASS3 showed similar and over twice the number of differentially expressed proteins compared with PASS1. We observed increased expression of drug-resistance-related proteins in PASS1−3 strains in both growth mediums, which was confirmed using MIC antimicrobial assays. Our findings reinforced the concept that clinical isolates of *P. aeruginosa* show reduced expression of proteins in relation to motility and adhesion, which assists the pathogen to avoid elimination by host defense responses. This situation is quite different to that seen with the PAO1 strain that has been subcultured under laboratory conditions. Hence, the data presented here show that the adaptation strategies of *P. aeruginosa* are both multifactorial and combinatorial, brought about by genetic flexibility to adapt and colonize niche microenvironments.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.6b00058.

> Table S1. Summary of features of *P. aeruginosa* strains utilized in this study. Table S2. Volume of 100 mM amino acid stock solutions added into SCFM medium. Figure S1. Growth curves for *P. aeruginosa* PAO1, PASS1−3. Figure S2. Run-to-run reproducibility of 2D-Nano-LC iTRAQ-MS/MS identification. Figure S3. Alignment of PilA protein from PASS1−3 stains and PAO1. Figure S4. Representative MS/MS spectra of a few differentially expressed proteins. (PDF)
>
> Table S3: List of all the *P. aeruginosa* proteins identified and their abundances. (XLSX)
>
> Table S4: Comparison of differentially expressed membrane protein abundance (*n*-fold change). (XLSX)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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Chapter -6

TMT and SWATH proteomic analyses reveal adaptation dynamics of *Pseudomonas aeruginosa* to the hypoxic environment

Rationale:

This chapter describes an investigation of *P. aeruginosa* adaptation to hypoxic environments, as typically occurs in lungs of cystic fibrosis (CF) patients. Two CF isolates (PASS2-3) and a laboratory strain (PAO1) of *P. aeruginosa* were cultured in lung nutrient mimicking condition (SCFM) under hypoxic stress and normal aerobic respiration condition to profile their proteomes using TMT-labelling and SWATH-MS. Comparison of proteome profiles under normoxic growth to hypoxic condition, for the respective strains, enabled to gain insights into how the *P. aeruginosa* proteomes adapts to a hypoxic environment. Additionally, this allowed us to comment on whether the general conclusions made from studies on PAO1 also apply to strains isolated from CF environment. We included PASS2-3 strains in this study since they were bearing unique genomic and phenotypic modulations different than PAO1. PASS1 was excluded from this study because previous analysis revealed high similarity to PAO1. Similarly, PASS4 was also excluded in this study due to its inability to grow in SCFM medium, under hypoxic conditions.

Contribution:

Bacterial cell culture, cell lysis, proteolytic digestion, mass spectrometry sample preparation, SWATH mass spectrometry, data analysis and interpretation was performed by me. TMT labelling and corresponding mass spectrometry data generation was performed by Dr. Joel Chick, at Harvard Medical School, Boston, USA. Entire chapter was written by me and read and approved by Prof. Mark Molloy and Dr. Christoph Krisp.

6.1 <u>Introduction:</u>

A hallmark of the autosomal recessive disorder, cystic fibrosis (CF) is the presence of highly viscous mucopurulent sputum in the lungs and a hampered mucociliary clearance (14). CF sputum is chemically complex and unique with nutrients which are often not detected in the healthy individuals (143). Bacteria enter and colonise the CF sputum and generate a steep oxygen gradient (116). Additionally, elevated consumption of oxygen by the epithelial cells collectively leads to either hypoxic (O_2<1%) or potentially anoxic conditions (116, 210). *P. aeruginosa* are frequently isolated from such hypoxic mucus plugs in high cell densities (up to 10^8 to 10^{10} CFU/ml) (141, 210) more so in the biofilm mode (127).

P. aeruginosa, being a facultative anaerobe, prefers respiration using oxygen as a terminal electron acceptor in the aerobic respiration as it yields the highest energy (211). Nonetheless, *P. aeruginosa* is capable of thriving under anaerobic conditions by switching to a dissimilatory nitrate respiration (denitrification) where nitrogen oxides, nitrate (NO_3^-) and nitrite (NO_2^-) are used as terminal electron acceptors in the respiratory chain (211). Furthermore, *P. aeruginosa* uses non-redox, arginine fermentation to generate ATP through conversion of arginine to ornithine with doubling time of ~40 hours (211). Lastly, pyruvate fermentation is employed for the anaerobic survival up to 18 days if denitrification is absent, however, it does not support any growth (119). Of note, respiration through denitrification pathways is unique to a few bacteria including *P. aeruginosa*, and absent in humans, which makes it a potential drug target to eradicate the challenging chronic infections associated with this pathogen.

Although there have been several genetic, biochemical and physiological studies focusing mechanism underpinning acclimatization of *P. aeruginosa* to hypoxic conditions of CF lungs (128, 212-215), the majority of them are limited to the study of the laboratory strain PAO1 and utilized general growth medium such as Luria broth, which poorly mimics the CF lung milieu (216). *P. aeruginosa* genomic and phenotype characterization conducted by us (138), along with other independent studies (217,

218), reported, CF isolates have unique molecular make-ups and adaptation strategies to flourish in the CF lungs, different to that of laboratory-adapted strain such as PAO1. Additionally, studies with PAO1 alone, may not principally represent *P. aeruginosa* population dynamics during chronic infection in CF, while CF isolates display considerable diversity in the adaptation strategies including, virulence factors, drug resistance, among others even when strains are isolated longitudinally from the same individual (11, 219).

An understanding of the complex repertoire of proteins that control anaerobic respiration by sensing the surrounding niche to ensure survival is essential to understanding the pathogenicity of *P. aeruginosa* (220). This study aimed to understand the role of global cellular proteins of *P. aeruginosa* in the adaptation and survival under oxygen limiting (hypoxic) conditions in the CF lungs. In this light, a global, quantitative whole cell proteome profiling of the *P. aeruginosa* CF strains (PASS2 and PASS3) grown in CF lung nutrient mimicking condition (SCFM) along with hypoxic stress (O_2=0.8 to 1%, denoted as O_2<1% henceforth) was performed and the protein expression profiles were compared with well aerated (O_2 ~21%(221)), normoxic growth using two complementary quantitative proteomics workflow; SWATH-MS and TMT-MS. Given the differences in the genome and phenotypes in PASS strains, we asked whether the general conclusions made by studies on PAO1 also apply to the CF strains or if they have a unique proteome expression profile rendering an advantage in the colonisation. Overall, this study attempted to provide a closer picture of "pathoadaptation" of *P. aeruginosa* in the CF lungs which is essential to limit its growth and eradicate using new therapeutic interventions.

6.2 Experimental methods:

6.2.1 Bacterial strains and culture conditions

P. aeruginosa strains PASS2, PASS3 previously isolated from the sputum of the CF patients (138) and reference strain PAO1 (ATCC 15692), were grown on solid Luria-Bertani (LB) agar, out of a frozen stock. Colonies of each strain were inoculated in the synthetic cystic fibrosis medium (SCFM) and grown aerobically overnight, at 37°C

and constant shaking at 150 rpm. The SCFM was prepared as explained in Kamath *et al* (222).

For the growth under hypoxic conditions (O2<1%), an overnight cultures of all strains were diluted 1:50 and inoculated into Erlenmeyer flasks sealed with rubber stoppers through which tubing from the gas mixer was inserted. Nitrogen gas was supplied through the inlet pipe for 1-2 h until the concentration of oxygen is ≤1% (Ranging 0.8 to 1%, denoted as O_2<1% henceforth) representing hypoxic or anoxic growth conditions (determined by Activon® (Model 401) dissolved oxygen meter). For the normoxic growth (O_2~21%) of the bacteria, overnight cultures of all strains were diluted 1:50 and inoculated into 50ml SCFM medium in a 200ml flask, with cotton plugs covering the mouth of the flask ensuring continues the exchange of gases. All the cultures were grown in biological triplicates (n=3), at 37°C and constant shaking at 150 rpm till late logarithmic phase. Cell pellets were collected by brief centrifugation at 2500g for 10 min at 4°C and washed thrice with phosphate-buffered saline (PBS), pH 7.4. The cell pellet was stored in -20^{0}C until use.

6.2.2   Protein extraction and In-solution digestion:

The cell pellet was resuspended in lysis buffer containing 50mM Tris-HCl (pH 8.5), 1% SDS (w/v), 8M urea, EDTA-free protease inhibitor cocktail (Roche, Germany), and acid washed glass beads and lysed by bead beating using a FastPrep FP120 bead-beater (Savant, USA). Centrifugation at 2500g was performed for 8 min at 4°C to separate cell debris, followed by brief sonication to degrade the intact DNA. Supernatant were processed as described by Chick *et al* (223) with modification. Briefly, samples were reduced with 10mM dithiothreitol (DTT) for one hour at room temperature followed by alkylation with 30 mM iodoacetamide (IAA) for 30 min at room temperature in the dark. The reaction was quenched with excess DTT for 15 min. Samples were then methanol-chloroform precipitated and protein pellet were subsequently washed twice with ice-cold methanol and dried. The protein pellet was reconstituted in sample buffer; 8M urea in 50mM Tris-HCl (pH 8.8). After dissolving the pellet, samples were diluted with 50mM Tris-HCl (pH 8.8) to bring the

concentration of urea to 1.6M followed by protein concentrations determined by micro-BCA assay (Pierce). Samples were digested at 37^{0}C, overnight with LysC protease at a 1:250 (protease: protein) ratio followed by digestion at 37^{0}C, for 8h with trypsin at a 1:250 (protease: protein) ratio. The digests were quenched with formic acid and samples were desalted using a C18 spin column (Sigma-Aldrich, USA) column and samples were divided into two equal fractions for SWATH-MS and TMT labelling and dried down by vacuum centrifugation. Peptide concentration of each sample was determined by micro-BCA assay (Pierce) prior to MS analysis.

6.2.3   SWATH-MS measurements

Each sample was analysed on a TripleTOF 5600 mass spectrometer in two stages; information-dependent acquisition-MS (IDA-MS) analysis for ion library generation, followed by SWATH-MS (Data independent acquisition-DIA) for label-free quantification.

6.2.3.1   Information dependent acquisition-MS and ion library generation:

For IDA-MS, biological triplicate samples of each respective strain were pooled and separated by reverse phase (RP) with a linear gradient of 5-40% of solvent B (90% v/v ACN 0.1% v/v FA) over 120 min at a flow rate of 600 nL/min using a nano-LC system (Eksigent, part of SCIEX) in conjunction with a TripleTOF™ 5600 (SCIEX) using positive nanoflow electrospray analysis. MS1 spectra were collected in the range 350-1500 m/z for 200ms. MS/MS spectra of 20 most intense m/z values exceeding a threshold >150 counts per second (cps) with charge stages between 2+ and 4+ were collected. Ion libraries were generated with ProteinPilot™ software 5.0 using the Paragon™ algorithm (SCIEX) thorough ID mode including biological modifications (224). MS/MS data were searched against the *Pseudomonas aeruginosa* strain PAO1 protein sequence database retrieved from GenBank (release; December 2012) and in-house generated *in-silico* translated genome databases of PASS2-3 strains. Carbamidomethylation of Cys residues was selected as a fixed modification. An Unused Score cut-off was set to 1.3 (95% confidence), equivalent to a protein false discovery rate (FDR) < 1%.

6.2.3.2   SWATH™ MS analysis (DIA-MS):

For SWATH-MS (DIA-MS), proteolytic peptides of the individual sample were separated over RP linear gradient of 5-40% of solvent B over 60 min at a flow rate of 600nL/min using the same LC and MS instruments as specified above with positive nanoflow electrospray mode. SWATH-MS acquisition was performed in a 60 variable m/z window method over a range of 400–1250 m/z selected based on the intensity distribution of precursor m/z in the IDA data sets. Collision energies were calculated for 2+ precursors with m/z values of lowest m/z +20 % for each window width and a collision energy spread of 5 eV was used.

Ion libraries of individual strains were imported into PeakView™ software 2.1 using the SWATH MicroApp 2.0 (SCIEX, release 25 August 2014) and matched against SWATH-MS data of individual replicate. Retention calibration was performed using endogenous peptides. Further, data was processed using following settings; maximum of 100 peptides per protein and six transitions per peptide, peptide confidence threshold of 99%, transition FDR<1%, 5 min extraction window, fragment extraction tolerance of 75 ppm and exclusion of modified peptides. Cumulative protein areas from extracted ion chromatogram were exported to Excel or Perseus 1.5.2.6 (172) for further analysis.

6.2.4   TMT labelling and mass spectrometry (TMT-MS):

6.2.4.1   TMT-labelling and sample fractionation:

Peptide pellets were reconstituted in 200 mM EPPS buffer (pH 8) and labelled with corresponding TMT 6-plex reagent™ (Thermo Scientific) as per manufacturer's instructions and incubated for 2hrs at room temperature. The reaction was quenched using hydroxylamine for 15 minutes. Peptides were acidified using 1% formic acid and respective samples were pooled in a 1:1:1:1:1:1 ratio. In total, 3 x 6-plex experiments corresponding to biological triplicates of hypoxic vs control of each strain were performed. Each of the 3 TMT experiments was fractionated by basic reversed-phase isocratic elution using a reverse phase cartridges (Pierce). Samples were loaded onto the reverse phase cartridges and elution was performed using 12

fractionation steps with the increasing acetonitrile concentrations according to the manufacturer's instructions. Fractions were then consolidated down to six fractions, dried and then desalted using Stage tips.

6.2.4.2 Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS)

Fractionated peptide samples were analysed using an Orbitrap Fusion Tribrid-MS (Thermo Scientific, USA) equipped with an ultra-high pressure liquid chromatography unit (Proxeon). Peptides were separated on a 3hr RP gradient of 6-30% solvent B (acetonitrile in 0.125% formic acid) gradient with a flow rate of ~400nl/min. In each data collection cycle, one full MS scan (400-1400 m/z) was acquired in the Orbitrap (1.2×10^5 resolutions and an AGC of 2×10^5 ions). The subsequent MS2-MS3 analysis was conducted for top 10 most intense ions and were fragmented by CID with following settings; collision energy of 35%, AGC 4×10^3, isolation window 0.5 Da, maximum ion accumulation time 150 ms with a dynamic exclusion for 40 seconds.

During the MS3 analyses for TMT quantification, precursors were isolated using a 2.5 Da m/z window and fragmented by 35% CID in the ion trap. Multiple fragment ions (SPS ions) were co-selected and further fragmented by HCD. Precursor ion selection was based on the previous MS2 scan and the MS2-MS3 was conducted using sequential precursor selection (SPS) methodology (169). HCD used for the MS3 was performed using 55% collision energy and reporter ions were detected using the Orbitrap with a resolution of 60,000, an AGC setting of 150,000 ions and a maximum ion accumulation time of 150ms.

6.2.4.3 Database searching and reporter ion quantitation

In-house software tools were used to convert RAW files to the .mzxml format (225). Erroneous charge state and monoisotopic m/z values were corrected as detailed in Huttlin *et al* (225). MS/MS spectra assignments were made with the Sequest algorithm (159) using an indexed database generated by combining the PAO1 protein sequence database retrieved from GenBank (release; December 2012) and in-

house generated *in-silico* translated genome databases of PASS2-3 strains (138) prepared with forward and reversed sequences concatenated according to the target-decoy strategy (161). All searches were performed using a static modification for cysteine alkylation and TMT on the peptide N-termini and lysines residues. Methionine oxidation was considered a dynamic modification, precursor ion tolerance of 20 ppm and a fragment ion tolerance of 0.8 Da (for CID) were used. Sequest matches were filtered by linear discriminant analysis as described previously (161), first to a dataset level error of 1% at the peptide level based on matches to reversed sequences. Peptide probabilities were then multiplied to create protein rankings and the dataset was again filtered to a final dataset level error of 1% FDR at the protein level. The final peptide-level FDR fell well below 1% (~0.2% peptide level). Peptides were then assigned to protein matches using a reductionist model, where all peptides were explained using the least number of proteins.

Peptide quantitation using TMT reporter ions was accomplished as previously published (168, 169). Briefly, a 0.003 Da m/z window centered on the theoretical m/z value of each reporter ion was monitored for each of the 6 reporter ions, and the intensity of the signal closest to the theoretical m/z value was recorded. TMT signals were also corrected for isotope impurities as per manufacturer's instructions. Peptides were only considered quantifiable if the total signal-to-noise for all channels was >200 and an isolation specificity of >0.75. Within each TMT experiments, peptide quantitation was normalised by summing the values across each channel and then each channel was corrected so that each channel had the same summed value. Protein quantitation was performed by summing the signal-to-noise for all peptides for a given protein. Protein quantitation values were exported for further analysis in Excel or Perseus 1.5.2.6 (172).

6.2.5 Statistical analysis

The data processing and statistical analysis of SWATH and TMT data was performed on Perseus 1.5.2.6 (172). Quantitative values were log-transformed and normalised based on the median value in each of the 18 samples. A pairwise comparison was

done by two-sided *t*-test and differentially expressed proteins were identified with the following cut-off; log$_2$ fold change of ±1 and FDR corrected *p*-value < 0.05. Fold changes are expressed as a ratio of the proteins present in hypoxic condition with that of normoxic values. Differential abundance values for individual proteins are expressed as log$_2$ transformed values.

6.3 Results:

6.3.1   Rationale and experimental design

The aim of this study was to discover proteins that potentially underpin the adaptation of *P. aeruginosa* to the hypoxic conditions in CF lungs. In this light, quantitative global whole cell protein expression profiles of three strains of *P. aeruginosa* (PASS2-3, PAO1) cultured in SCFM under hypoxia (Oxygen supply <1%) were compared with respective aerobically grown (normoxia) strains as controls. PASS strains used in this study were isolated from sputum of the CF patients of diverse age, gender, CFTR gene mutation and antibiotic treatment groups and the genome and phenotypic characteristics were recently reported by us (138). Palmer *et al* demonstrated the nutrient conditions of the SCFM closely resembled CF patient's sputum, and the growth pattern of *P. aeruginosa* when grown in CF patient's sputum, closely resembled the growth in SCFM (141). Hence, we rationalised that the growth conditions and strains used in this study closely mimic conditions of the CF lungs.

Of note, all strains of *P. aeruginosa* grew well in SCFM under hypoxic condition. However, all the strains showed slower growth under hypoxic conditions compared to the normoxic growth, indicating oxygen is a growth limiting factor.

6.3.2   *P. aeruginosa* protein quantification using a combination of multiplexed TMT and SWATH-MS strategies.

Recently, the TMT-labelling proteomic approach has been widely used in large-scale discovery proteomic studies due to the robustness of the method and capacity of multiplexing up to 10 samples (226, 227). Additionally, SWATH-MS has been a method of choice in quantitative analysis of complex proteome due to excellent run to run reproducibility and wider magnitude of dynamic range (170, 228). This study

combines the quantitative capacity of SWATH and TMT to generate the comprehensive quantitative proteome profiles of the *P. aeruginosa*.

Using the method as outlined in Figure-6.1, proteolytically cleaved whole cell proteins of *P. aeruginosa* were analysed in biological triplicates using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and quantified using TMT labelling and SWATH-MS approaches.

In SWATH-MS quantification, by using a peptide confidence threshold of 99%, transition FDR<1%, we unambiguously quantified 2033 proteins (Supplementary data file 6A) collectively in three strains. When the same biological samples were subjected to TMT-MS analysis, by applying FDR of <1% both at peptide and protein levels we unambiguously quantified 3849 proteins (Supplementary data file 6A) collectively in three strains. Integrating results from both techniques, enabled quantification of 3967 proteins (Supplementary data file 6A), collectively in PAO1, PASS2and PASS3 across all biological triplicates, which represents extensive proteome coverage (~71% of all predicted ORFs of *P. aeruginosa* strain PAO1). This is an approximate two-fold increase in the number of *P. aeruginosa* proteins (2100 proteins) reported by others in CF (AES-1R) and other reference strains (PAO1, PA14) grown in LB and artificial sputum medium (151). Thus, the current study provides the most comprehensive proteomic analysis of *P. aeruginosa* whole cellular proteins reported so far.

High run to run reproducibility was observed between biological replicates, both in SWATH and TMT experiments (Figure-6.2A, B). All the quantified proteins were clustered using the Euclidean distance metric and principle component analysis. As expected, the biological triplicates clustered together, while the hypoxic and normoxic samples of respective strains clustered closer to each other (Figure-6.2D, E), rather than strains subjected to hypoxia clustering together. Comparing the number of proteins identified in each technique, approximately 1800 proteins were commonly quantified between SWATH and TMT techniques in each strain. Over 150 and 1450 proteins were identified uniquely in SWATH and TMT approach

respectively, in each strain (Figure-6.2C). Increased identification of proteins reported with the TMT-labelled samples is due to differences in sample acquisition which included off-line peptide fractionation using high pH reverse phase chromatography, peptide separation on a longer RP column (30cm) and mass spectrometer detection capacity (229).
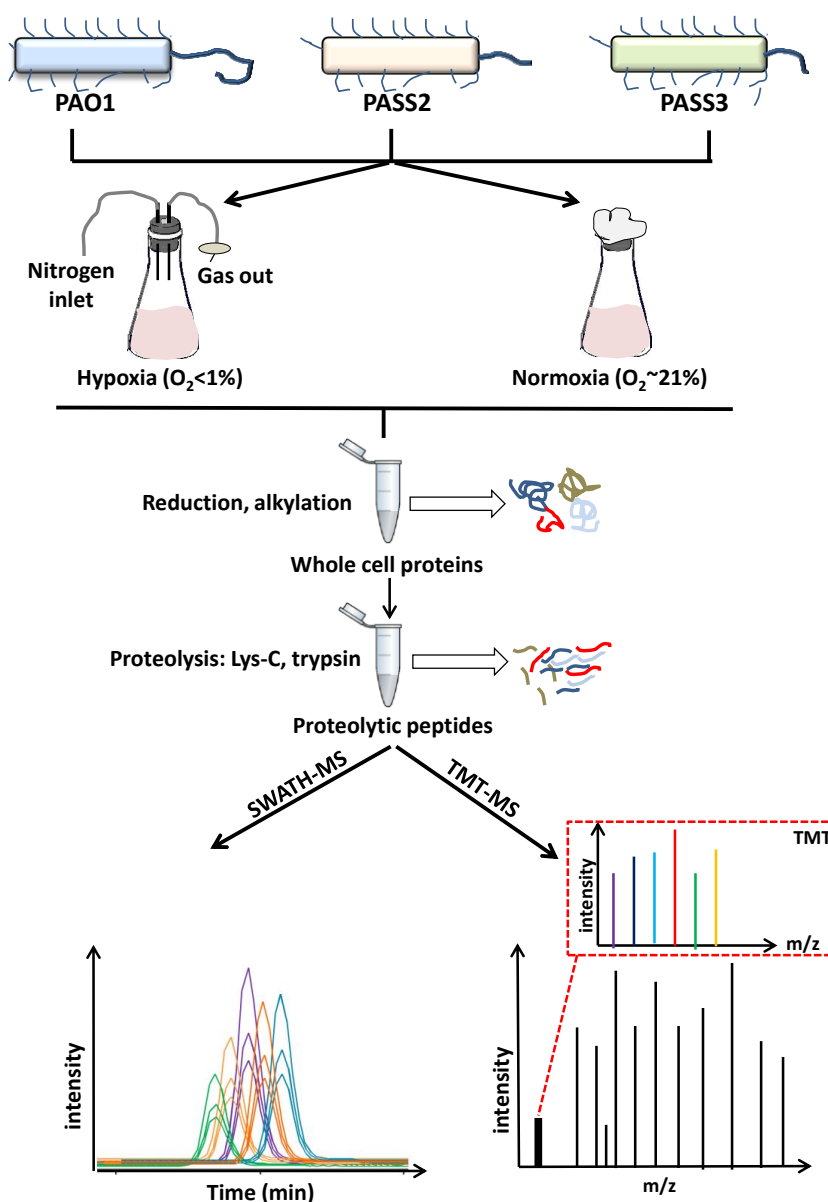
Figure-6.1: Analysis of *P. aeruginosa* proteins using complimentary TMT and SWATH MS techniques. *P. aeruginosa* strains PAO1, PASS2 and PASS3 were grown in hypoxic and normoxic conditions in biological triplicate. Cells were lysed and whole cellular proteins were subjected to reduction, alkylation and Lys-C and trypsin digestion. Proteolytic peptides were labelled with TMT reagents and subjected to LC-MS/MS analysis. Additionally, peptides were subjected to LC-MS/MS and quantified using SWATH-MS.

6.3.3 Evaluation of *P. aeruginosa* proteome quantitation methods-TMT and SWATH strategy:

For the identification of differentially expressed proteins, we compared the $\log_2$ expression values of proteins in hypoxic condition with those corresponding in normoxic condition (*n*=3) using a two-tailed *t*-test and generated a corrected *p*-value with a cut-off ≤ 0.05. Additionally, we imposed a second threshold requiring $\log_2$ fold change cut-off ±1. Of note, the fold-change reporting threshold used in this study is different to that used in our previous work (Chapter-5), comparing membrane proteomes of *P. aeruginosa* strains using iTRAQ-MS, where a fold-change cut-off of ±1 was used, to circumvent the issue of ration compression caused due to iTRAQ based quantification. Pervious work by Ow (230) demonstrated, while analysing complex samples, fold change obtained by iTRAQ suffers to some extent from the compression of the quantitation ratios to a ratio of one. Consequently causing an under-estimation of the ratios, although the general trends of up or down regulation are correct. However, such issues of ratio compression are virtually absent in the case SWATH based quantification, hence eliminating the need for using a lower fold-change cut-off. The proteins up and down-regulated for each strain are summarised in Table-6.1 and Supplementary data file 6A.

Table 6. 1: SWATH and TMT-MS identification of differentially abundant proteins (hypoxic versus normoxic) in *P. aeruginosa* PASS2, PASS3 and PAO1 cultured in SCFM.

Strain	Total identified proteins	Identified SWATH	Identified TMT	Differentially expressed proteins#	Differentially expressed in SWATH#	Differentially expressed in TMT#
PAO1	3631	2033	3489	735	322	572
PASS2	3496	2033*	3289	640	317	460
PASS3	3523	2033*	3330	364	228	175

*Indicates the PASS2 and PASS3 proteins identified by searching the data against PAO1 ion library. # $\log_2$ fold-change cut-off ±1, t-test *p*-value <0.05.

We next evaluated the correlation between quantification by TMT and SWATH. Figure-6.2F-H plots the ratio of differentially expressed proteins (in log$_2$) obtained by TMT against that by SWATH. We observed an excellent correlation in ratios derived from TMT and SWATH-MS with Pearson correlation coefficient of 0.96, 0.94 and 0.87 and *p*-value (two-tailed) < 0.0001 for PAO1, PASS2 and PASS3 respectively (Figure-6.2F-H). This indicates an excellent agreement in quantitative ratios determined by two independent techniques, SWATH and TMT. The correlation found in this study is better than those reported in previous studies (231-233) comparing the quantitative capacity of iTRAQ with SWATH.

The improved correlation between SWATH and TMT in our study, in part, is likely attributed to SPS based MS3 scan, used for TMT reporter ion quantification, which almost completely eliminates the commonly encountered issue of underestimation of the ratios (168, 169). Additionally, unlike previous studies (231-233) where trypsin is used for the generation of proteolytic peptides, we used a combination of Lys-C and trypsin. This strategy increases the availability of C-terminal lysine residue and N-terminus for labelling with amine-reactive TMT reagents (168). Consequently, in the MS2, both *b*- and *y*-type fragment ions bear a TMT tag, and a MS3 scan on almost any fragment ion will produce TMT reporter ions.

Notably, PAO1 and PASS2 displayed more differentially expressed proteins compared to PASS3, and this was consistent in both SWATH and TMT techniques. Interestingly, we observed very moderate overlap in differentially expressed proteins between PAO1 and PASS strains with the most number of unique proteins in PAO1 (Figure-6.3). This is consistent with the previous report (138) where SWATH proteomic profiling showed more unique proteins in PAO1, while PASS strains shared relatively large proportion of proteins. This may indicate the heterogeneity in the protein expression between strains reflecting the genetic diversity between strains.

Figure-6.2:
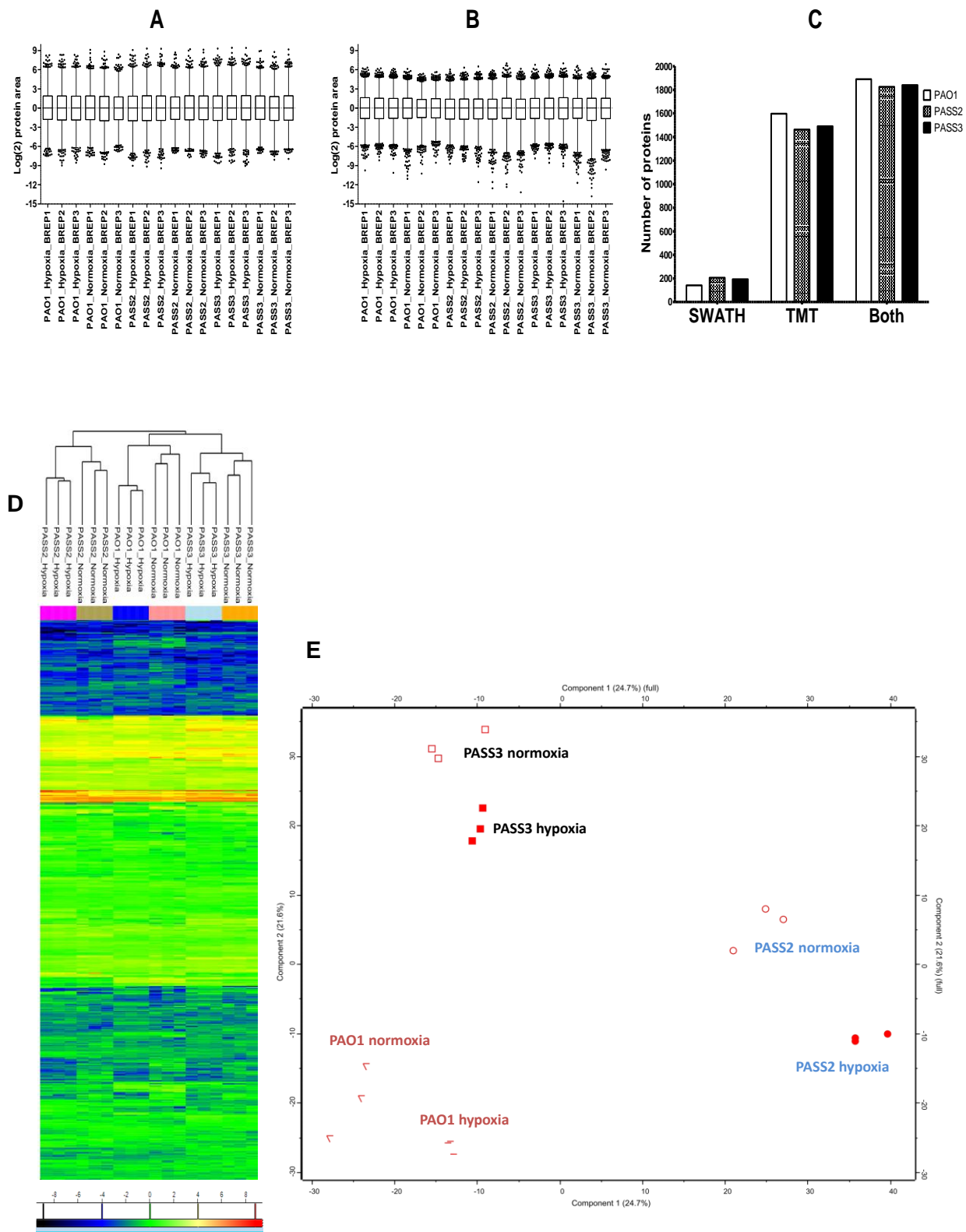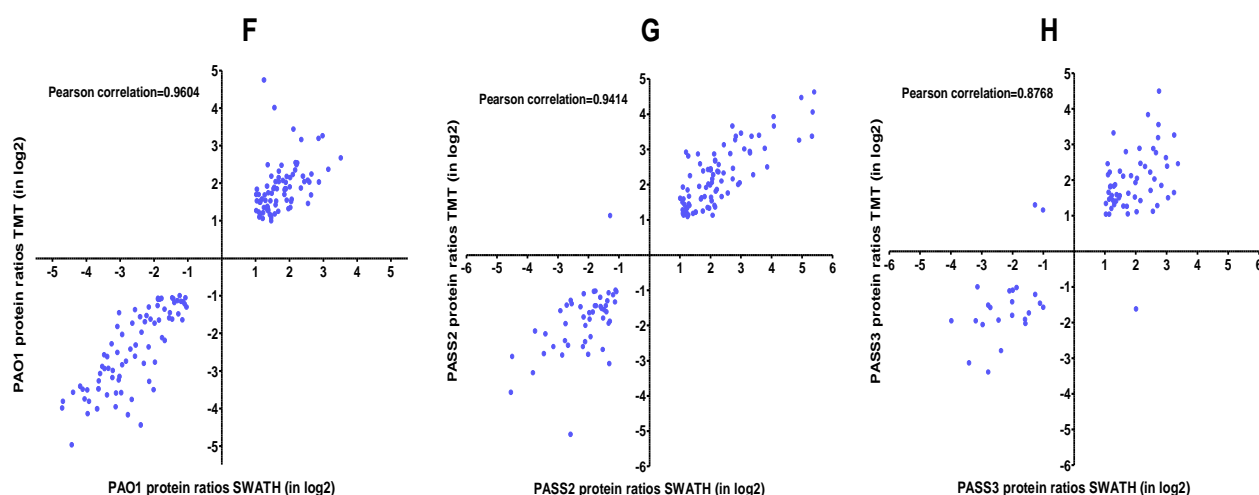
Figure-6.3: Evaluation of quantification techniques; TMT and SWATH MS. Box plots of log$_2$ transformed, normalized protein peak areas of individual biological replicates displaying reproducibility of (A) SWATH and (B) TMT. (C) Bar graph representing a comparison of number of common and unique PAO1 and PASS1-2 proteins identified in SWATH and TMT. (D) The hierarchical clustering and heat map of SWATH-MS log$_2$ protein expression data of PAO1, PASS2 and PASS3. (E) Strains of *P. aeruginosa* and different oxygen treatments classified based on log$_2$ transformed and normalized values using Principal component analysis (PCA). Each spot represents each biological replicate of PAO1, PASS2 and PASS3. Plots of the ratios of differentially expressed proteins (in log$_2$) obtained by TMT against SWATH in (F) PAO1, (G) PASS2 and (H) PASS3.
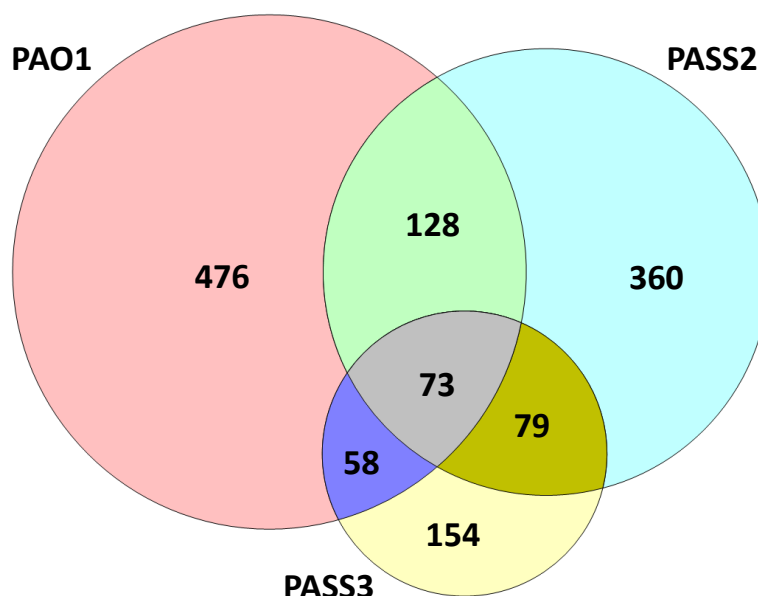
Figure-6.4: Features of differentially expressed *P. aeruginosa* proteins. Venn diagram representing the overlap of differentially expressed (hypoxic versus normoxic) proteins between PAO1, PASS2 and PASS3.

Among the proteins that were altered in abundance in one or more strain during growth in hypoxic conditions compared to the normoxic baseline were proteins needed for both aerobic and anaerobic respiration, iron acquisition, stress response and protein secretion system, among others, with considerable heterogeneity observed in the expression profile between strains (Supplementary data file 6A).

6.3.4   Terminal oxidases play an indispensable role in the adaptation of *P. aeruginosa* to the hypoxic condition

P. aeruginosa copes with the oxygen limitation in part through terminal oxidases which can be modulated in expression in response to external stresses including hypoxic stress (178). Of five known terminal oxidases in *P. aeruginosa*, cytochrome *cbb₃* type oxidases (*cbb₃*-1 and *cbb₃*-2) are widely studied due to their critical role in the adaptation to hypoxic conditions (234).

We quantified four proteins, each of which form the subunits of *cbb₃*-1 (PA1552-PA1554) and *cbb₃*-2 (PA1555-PA1557), and majority of the subunits of both oxidases were significantly elevated (1.3-5.1 fold) in all strains when grown under hypoxic condition (Table-6.2), while PASS2 *cbb₃* oxidases showed same or higher expression

compared to PAO1 and PASS3 (Supplementary Figure-9.10). Although both *cbb₃* oxidases were up-regulated in PASS2 on exposure to hypoxic condition, *cbb₃*-2 oxidase showed higher magnitude of up-regulation (3.4 to 5.1) compared *cbb₃*-1 (1.4 to 1.7 fold), indicating *cbb₃*-2 may play a major role in hypoxic respiration of the bacterium. Increased abundance of *cbb₃*-2 is consistent with the previous report that it is essential for the survival of *P. aeruginosa* under microaerobic condition (234). Higher oxygen affinities (234) and elevated expression of *cbb₃* oxidases on exposure to hypoxic condition, supports the view that these enzymes are pivotal in the survival of the bacterium under hypoxic milieu such as CF lungs.

Table 6.2: Differentially expressed terminal oxidases of *P. aeruginosa*, PAO1, PASS2-3, in response to hypoxia (O2<1%) compared to normoxia, determined by SWATH and TMT MS. Red and green shading indicates proteins up-regulated (Log$_2$ Fold change (FC)>1, *t*-test *p*-value<0.05) and down-regulated (Log$_2$ Fold change (FC) <1, *t*-test *p*-value<0.05) respectively. Locus tag refers to the corresponding PAO1 locus tag.

Locus tag	Gene	FC_PAO1 SWATH	FC_PAO1 TMT	FC_PASS2 SWATH	FC_PASS2 TMT	FC_PASS3 SWATH	FC_PASS3 TMT
PA1554	**ccoN1**	0.2	1.53	-0.87	1.72	0.64	0.55
PA1557	**ccoN2**	1.36	2.49	1.35	5.13	-0.03	ND
PA1553	**ccoO1**	-0.95	0.35	0.72	1.42	-0.52	1.14
PA1556	**ccoO2**	2.34	1.87	4.08	3.66	2.98	2.63
PA1552	**ccoP1**	0.48	0.74	-0.6	-0.17	0.01	0.13
PA1555	**ccoP2**	1.53	1.85	3	3.46	3.02	2.39
PA1552.1	**ccoQ1**	ND	0.33	ND	1.33	ND	0.39
PA1555.1	**ccoQ2**	0.38	ND	1.81	ND	0.75	ND
PA3930	**cioA**	0.76	1.18	2.29	2.05	-0.79	2.32
PA1317	**cyoA**	ND	-0.41	ND	ND	ND	-1.03
PA1318	**cyoB**	ND	-1.47	ND	ND	ND	-1.62

ND: Not detected, FC: Fold change (hypoxia/normoxia)

The remaining three *P. aeruginosa* terminal oxidases, namely *bo₃*-type quinol oxidase (Cyo), cyanide-insensitive oxidase (CIO), *aa₃*-type cytochrome c oxidase (*aa₃*) are reported to have a relatively low affinity to oxygen compared to *cbb₃* type oxidases (234). CIO-A (PA3930) was increased in abundance by ~2 fold in PASS2, but not significantly altered in other strains (Table-6.2). CIO was previously reported as induced under copper starvation and cyanide (178), however, no measurements for the production of cyanide by PASS2 was performed in this study. Another oxidase Cyo was down-regulated in PAO1 and not significantly changed in other strains.

Decreased abundance (4.4-9.6 fold) of Cyo, when grown in 0.4% oxygen compared to 20% oxygen, was previously shown in a transcriptome study in PAO1 grown in SCFM medium (128). Prior work looking at the expression of Cyo (179) indicated that the oxidase is essential during relatively high availability of oxygen and under iron starvation. Lastly, expression of *aa3*-oxidase was not significantly changed in any strain under hypoxic growth. In summary, of five terminal oxidases, *cbb3* oxidases were significantly induced in all strains and CioA was induced only in PASS2, indicating the indispensable role of *cbb3* oxidases in hypoxic respiration of *P. aeruginosa*.

6.3.5 Anaerobic respiration/denitrification proteins are simultaneously expressed when grown under hypoxia:

P. aeruginosa survives in an anaerobic environment in part through dissimilatory denitrification, using nitrogen oxides as an alternative terminal electron acceptor in the respiratory chain (6). 33 proteins were detected (Supplementary data file 6A) in relation to denitrification enzymes (Nar, Nap, Nir, Nor, Nos). On exposure to hypoxic stress, the majority of the enzymes were significantly elevated in PASS2 with much greater *n*-fold induction, while, induced at a lower level in PASS3 and either not induced or were induced at a lower level in PAO1, compared to normoxic baseline (Table-6.3). For example, we quantified all of four predicted subunits of nitrate reductase (Nar G, H, I, J). NarG-I were increased in abundance by 2-3 fold in PASS2 and not significantly altered in PAO1 and PASS3, except for NarI (2.3 fold) and NarG (1.08 fold) up-regulated in PAO1 and PASS3 respectively.

Enzymes involved in the arginine fermentation through the arginine deiminase pathway (ArcA, ArcB, ArcC) were elevated in expression in all strains with almost similar magnitude of expression (Table-6.3). Although alcohol dehydrogenase was found up-regulated in all strains during hypoxic growth, we did not observe any significant changes in other pyruvate fermentation pathway proteins. Increased abundance of alcohol dehydrogenase under hypoxic growth has been previously reported in the closely related Gram-negative bacterium *E. coli* (235).

Table 6.3: Differentially expressed anaerobic respiration and arginine fermentation related proteins of *P. aeruginosa,* PAO1, PASS2-3, in response to hypoxia (O2<1%) compared to normoxia, determined by SWATH and TMT MS. Red and green shading indicates proteins up-regulated (Log$_2$ Fold change (FC)>1, *t*-test *p*-value<0.05) and down-regulated (Log$_2$ Fold change (FC) <1, *t*-test *p*-value<0.05) respectively. Locus tag refers to the corresponding PAO1 locus tag.

Locus tag	Gene	FC_PAO1 SWATH	FC_PAO1 TMT	FC_PASS2 SWATH	FC_PASS2 TMT	FC_PASS3 SWATH	FC_PASS3 TMT
PA1173	**napB**	1.17	0.82	-0.87	2.33	1.27	0.96
PA1172	**napC**	-1.87	1.3	-1.59	ND	-2.4	0.82
PA3875	**narG**	1.14	ND	2.38	ND	1.08	ND
PA3874	**narH**	1.32	1.17	2.48	3.43	0.62	1.23
PA3872	**narI**	2.3	1.77	3.12	3.51	0.44	0.64
PA3873	**narJ**	ND	-2.64	ND	1.06	ND	-0.73
PA3877	**narK1**	ND	0.71	ND	1.9	ND	0.89
PA3876	**narK2**	0.54	0.62	-0.47	ND	-0.26	1.53
PA3879	**narL**	0.87	0.84	2.09	2.28	1.04	0.68
PA3878	**narX**	-0.17	0.7	0.88	1.67	1.22	0.59
PA1781	**nirB**	ND	ND	ND	3.05	ND	ND
PA0517	**nirC**	1.14	2.42	5.39	5.21	0.14	0.42
PA0515	**nirD**	ND	1.22	ND	3.45	ND	-0.27
PA0510	**nirE**	ND	0.01	ND	0.61	ND	0.52
PA0516	**nirF**	0.37	0.36	3.29	2.94	1.48	1.56
PA0513	**nirG**	-0.37	1.27	0.29	3.25	-1.75	0.69
PA0512	**nirH**	ND	0.02	ND	3.84	ND	1.31
PA0511	**nirJ**	-1.17	-0.06	-0.1	2.43	-1.27	1.3
PA0514	**nirL**	ND	0.1	ND	1.22	ND	-0.18
PA0518	**nirM**	0.05	0.97	5.33	3.37	0.56	-0.62
PA0509	**nirN**	-0.56	0	0.22	2.97	0.04	1.79
PA0520	**nirQ**	-0.72	1.38	1.66	2.89	0.12	2.1
PA0519	**nirS**	0.13	1.04	3.59	3.4	1.16	2.22
PA0524	**norB**	0.67	0.68	3.86	2.5	0.88	0.45
PA0523	**norC**	-0.02	ND	4.41	ND	0.68	ND
PA3393	**nosD**	ND	1.05	ND	2.48	ND	0.92
PA3394	**nosF**	ND	0.79	ND	3.58	ND	1.42
PA3396	**nosL**	1.75	2.89	1.52	ND	-0.5	3.03
PA3392	**nosZ**	1.06	1.36	2.5	2.97	1.42	2.45
PA5171	**arcA**	1.29	1.54	1.2	ND	1.8	0.83
PA5172	**arcB**	0.98	1.34	1.18	ND	1.7	0.77
PA5173	**arcC**	0.78	0.73	0.83	0.95	1.7	0.81
PA5170	**arcD**	0.7	1.55	0.12	1.73	-0.22	1.57

ND: Not detected, FC: Fold change (hypoxia/normoxia)

Collectively, these results confirm the concurrent induction of aerobic and anaerobic respiration, and arginine fermentation during hypoxic growth of *P. aeruginosa*, however, PASS2-3 showed a higher n-fold change of proteins involved in such pathways compared to PAO1.

6.3.6 _P. aeruginosa_ PAO1 displays decreased abundance of proteins involved in the iron acquisition pathway under hypoxic stress:

We quantified several proteins in relation to iron metabolism in all three strains, including siderophore biosynthesis, secretion system, transmembrane transport of iron bound siderophores and proteins that process iron bound siderophores and re-secrete them back to the external milieu. Strikingly, the majority of the proteins were significantly decreased in abundance on exposure to hypoxic compared to normoxic growth, predominantly in PAO1, while, the majority of such proteins were either unaltered, not detected or also down-regulated in PASS2-3 (Table-6.4, Supplementary data file 6A).

The lack of detection or differential expression of iron acquisition proteins, for instance, siderophores, in PASS2 and PASS3 is consistent with our previous observation (138) that both strains produced little or no pyoverdine. Furthermore, genome analysis revealed, PASS2 had complete or partial loss of multiple pyoverdine biosynthesis and receptor genes resulting in the absence of pyoverdine biosynthesis (138).

The abundance of iron scavenger siderophores, pyoverdine and pyochelin (chelator of (Fe^{3+}) iron) was altered in expression in all strains on exposure to hypoxic condition. A significant reduction in the abundance of many proteins involved in biosynthesis and secretion of pyochelin (PchA-G, Table-6.4) was observed equally in PAO1 and PASS3, while PASS2 showed little change or not significantly altered in the expression compared to normoxic baseline. Pyoverdine biosynthesis proteins were found decreased in abundance in PAO1, but unaltered or down-regulated in PASS strains (Table-6.4, Supplementary data file 6A). The data indicates, PAO1 dramatically reduces expression of both pyoverdine and pyochelin on exposure to a hypoxic condition, while the expression in PASS strains was less severe or unaltered upon exposure to hypoxic conditions.

Table 6.4: Differentially expressed iron metabolism related proteins of *P. aeruginosa*, PAO1, PASS2-3, in response to hypoxia (O2<1%) compared to normoxia, determined by SWATH and TMT MS. Red and green shading indicates proteins up-regulated (Log$_2$ Fold change (FC)>1, *t*-test *p*-value<0.05) and down-regulated (Log$_2$ Fold change (FC) <1, *t*-test *p*-value<0.05) respectively. Locus tag refers to the corresponding PAO1 locus tag. Genes marked in red represent the predicted TonB dependent iron transporters of *P. aeruginosa* PAO1 (236).

Locus tag	Gene	FC_PAO1 SWATH	FC_PAO1 TMT	FC_PASS2 SWATH	FC_PASS2 TMT	FC_PASS3 SWATH	FC_PASS3 TMT
PA2385	**pvdQ**	-3.64	ND	-0.98	ND	-0.41	ND
PA2386	**pvdA**	-4.39	-3.57	0.48	1.81	-0.83	-0.33
PA2391	**opmQ**	ND	-2.02	ND	-3.59	ND	ND
PA2392	**pvdP**	ND	-2.06	ND	ND	ND	ND
PA2394	**pvdN**	-2.25	-2.87	0.1	ND	-0.52	ND
PA2395	**pvdO**	-2.36	-3.28	0.12	0.76	1.05	-0.34
PA2396	**pvdF**	-2.54	-2.31	0.94	2.57	0	0.7
PA2397	**pvdE**	ND	-3.37	ND	0.06	ND	1.01
PA2399	**pvdD**	-3.02	-3.15	-0.37	0.16	-0.59	-0.45
PA2400	**pvdJ**	ND	-3.02	ND	1.97	ND	ND
PA2413	**pvdH**	-1.68	-2.19	0.46	0.94	1.05	ND
PA2424	**pvdL**	-2.16	-2.87	-1.3	1.22	0.24	-0.97
PA4224	**pchG**	-2.83	-2.74	-1.2	-1.06	-2.37	-0.84
PA4225	**pchF**	-3.63	-3.27	-0.88	-1.38	-2.27	-0.94
PA4226	**pchE**	-3.97	-3.51	-0.75	-1.11	-1.99	-0.48
PA4227	**pchR**	ND	-2.79	ND	-1.09	ND	-0.69
PA4228	**pchD**	-2.81	-3.27	-1.44	-1.77	-2.18	-0.97
PA4229	**pchC**	-3.38	-2.62	-0.06	-0.48	-3.14	-1
PA4230	**pchB**	-4.18	-3.41	0.95	-0.27	-1.43	-0.18
PA4231	**pchA**	-3.52	-2.56	-0.77	-1.25	-1.88	-0.97
PA2398	**fpvA**	-2.7	-2.42	-1.87	0.7	-2.01	0.17
PA4168	**fpvB**	-4.78	ND	1.89	ND	-1.36	ND
PA4221	**fptA**	-2.98	ND	2.22	ND	-0.64	ND
PA0931	**pirA**	ND	-2.06	ND	0.45	ND	ND
PA2388	**fpvR**	ND	0.13	ND	0.29	ND	2.2
PA1910	**femA**	ND	-1.14	ND	0.11	ND	ND
PA2466	**foxA**	ND	-2.22	ND	0.3	ND	-1.45
PA4708	**phuT**	-2.3	-2.8	0.09	0.12	-1.2	-0.63
PA4709	**PhuS**	-2.66	-3.76	0.45	0.73	-1.55	-0.89
PA4710	**phuR**	-2.16	-2.36	2.47	1.83	-1.04	-1.1
PA0672	**HemO**	-3.65	-2.2	3.06	0.83	-2.41	-0.98
PA1271	**PA1271**	-0.51	0.55	-1.32	0.97	-1.9	-0.78
PA1302	**PA1302**	ND	-2.35	ND	-0.07	ND	-0.04
PA1922	**PA1922**	-3.96	-4.14	-1.52	-1.79	-1.39	-2.81
PA0781	**PA0781**	-3.59	-3.07	-0.38	0.11	-1.77	-0.51
PA4358	**PA4358**	ND	-3.32	ND	2.65	ND	0.56
PA4515	**piuC**	-2.79	-2.59	0.3	-1.01	-1.35	-1.06
PA4675	**PA4675**	-1.83	-1.85	-1.53	-0.96	-1.55	-1.14
PA4687	**hitA**	-1.81	-0.5	0.02	0.23	-0.71	-0.99
PA4688	**hitB**	ND	ND	ND	-2.11	ND	-0.56

Locus tag	Gene	FC_PAO1	FC_PAO1	FC_PASS2	FC_PASS2	FC_PASS3	FC_PASS3
		SWATH	TMT	SWATH	TMT	SWATH	TMT
PA4837	**PA4837**	-3.23	-3.18	-0.46	0.02	-1.08	-2.26
PA5531	**tonB**		-2.46		-0.36		-0.92
PA1927	metE	-3.92	-3.81	-0.19	2.77	-1.37	0.91
PA2393	**PA2393**	-0.87	-2.68	0.07	-0.55	0.29	ND
PA2402	**PA2402**	-3.64	-3.48	-0.13	ND	0.18	0.04
PA2911	**PA2911**	-1.58	-1.64	-1.3	-0.13	-0.66	-2.35
PA3268	**PA3268**	0.01	-1.53	0.13	0.14	-0.22	-0.97
PA3866	**PA3866**	ND	-1.45	ND	0.36	ND	1.45

ND: Not detected, FC: Fold change (hypoxia/normoxia)

Furthermore, receptors for ferripyoverdine (FpvA, FpvB), ferripyochelin (FptA) and receptors for heterologous iron sources, including, PirA for enterobactin, FemA for ferric-mycobactin and FoxA for ferrioxamine B (237, 238) were decreased in abundance, on exposure to hypoxic conditions, predominantly in PAO1. Out of 34 predicted Ton-B dependent receptors in PAO1 (236), we quantified 25 and the majority of them were decreased in abundance predominantly in PAO1 (genes highlighted in red in Table-6.4), while showing little or no significant change in PASS strains.

P. aeruginosa also assimilates iron through heme using hemoproteins via the Has and Phu system (237). Heme intake in *P. aeruginosa* PAO1 is through two outer membrane receptors, PhuR (*Pseudomonas* heme uptake) and HasR (heme assimilation system) (239). On exposure to hypoxic growth, PhuR was found ~2.3 and 1.1 fold down-regulated in PAO1 and PASS3 but not significantly altered in PASS2 strain. PhuT, the only periplasmic heme binding protein described in *P. aeruginosa* (239), was down-regulated in PAO1 and PASS3. Heme in the cytoplasm binds to PhuS (PA4709) which delivers it to HemO (PA0672) for degradation to biliverdin and Fe^{2+} (239). Interestingly, both PhuS and HemO were down-regulated in PAO1 and PASS3 and not significantly changed in PASS2 during hypoxic growth. *P. aeruginosa* assimilates ferrous iron (Fe^{2+}) through a dedicated outer membrane channel Feo (PA4358) (239), and the protein was reduced in abundance in PAO1 under hypoxic growth and unaltered in PASS strains.

In summary, hypoxic stress triggered down-regulation of proteins associated with both ferrous and ferric iron acquisition, predominately in PAO1, while PASS strains showed reduction of expression of selective pathways, such as pyochelin biosynthesis proteins in PASS3.

6.3.7 *P. aeruginosa* displays a robust stress response to hypoxic condition:

Several proteins essential for the response to the external stress were increased in abundance in all strains under hypoxic conditions (Table-6.5, Supplementary data file 6A). Among those, a periplasmic protein, cytochrome c551 peroxidase (CcpR), was found increased 2.7-5 fold in abundance in all strains (Supplementary Figure-9.11). Although the physiological role of CcpA is not established in *P. aeruginosa* the enzyme catalyses the conversion of H_2O_2 to water and it is predicted to be induced under hypoxic conditions (128). Nonetheless, it is logical to expect that it might assist in cellular detoxification as established in *Rhodobacter* and yeast (47). Another enzyme involved in the protection from oxidative damage, catalase (KatA) was upregulated equally (1-1.5 fold) in all strains under hypoxic growth. Interestingly, another catalase, KatB, was down-regulated in all three strains under hypoxic growth. KatA is known to be constitutively expressed under oxidative stress; however KatB is detectable only when the cells are exposed to peroxide or paraquat (240), which might explain the reduced expression of KatB observed here. Interestingly, PA3309 and PA4352 are two predicted universal stress proteins and both were up-regulated in PASS2 and PASS3 but not significantly changed in PAO1. Although the precise function of these proteins remains unknown, they have been implicated important for survival during anaerobic pyruvate fermentation (241) and anaerobic energy stress (242). Expression of stringent starvation protein A (SspA, PA4428) was elevated in PAO1 and PASS2 but did not significantly alter in PASS3. SspA protein has high sequence similarity with *E. coli* SspA which has been characterised as essential for survival under starvation (243). Lastly, on exposure to hypoxic conditions superoxide dismutase protein (SodB) was increased in abundance in all strains in a similar magnitude (1-1.5 fold). In summary, the study quantified several genes previously known to be induced (directly or indirectly) by oxidative stress in

P. aeruginosa and thus validates the applicability of proteomic experiments to reveal candidate proteins important for oxidative and hypoxic stress adaptation.

Table 6. 5: Differentially expressed stress response related proteins of *P. aeruginosa*, PAO1, PASS2-3, in response to hypoxia (O2<1%) compared to normoxia, determined by SWATH and TMT MS. Red and green shading indicates proteins up-regulated (Log$_2$ Fold change (FC)>1, *t*-test *p*-value<0.05) and down-regulated (Log$_2$ Fold change (FC) <1, *t*-test *p*-value<0.05) respectively. Locus tag refers to the corresponding PAO1 locus tag.

Locus tag	Gene	FC_PAO1 SWATH	FC_PAO1 TMT	FC_PASS2 SWATH	FC_PASS2 TMT	FC_PASS3 SWATH	FC_PASS3 TMT
PA4587	**ccpR**	2.06	2.99	4.98	4.47	2.74	3.56
PA4236	**katA**	1.35	1.29	1.09	1.28	1.17	1.5
PA4613	**katB**	ND	-2.71	ND	-1.73	ND	-1.7
PA3309	**PA3309**	0.7	0.81	3.4	2.28	1.76	1.63
PA4352	**PA4352**	0.84	0.98	2.73	2.16	2.3	2.38
PA4428	**sspA**	1.25	0.74	1.08	1.5	-0.12	0.32
PA0962	**PA0962**	0.97	1.5	1.24	1.34	1.3	1.31
PA3529	**PA3529**	1.69	2.04	1.14	0.74	0.62	0.43
PA3529	**PA3529**	1.69	2.04	1.14	0.74	0.62	0.43
PA4366	**sodB**	0.94	1.48	1.18	1.29	1	0.58

ND: Not detected, FC: Fold change (hypoxia/normoxia)

6.3.8 Differential expression of protein secretion systems in response to hypoxic stress:

Structural components of type I secretion system (T1SS) (AprD, AprE, AprF) and exoprotein AprX were decreased in abundance in PAO1, while, in PASS2 and PASS3 either not induced or not significantly altered in expression. Notably, the majority of proteins in relation to type 6 secretion system (T6SS) (PA0076-PA0091) were almost exclusively up-regulated in PASS3, however, such proteins were unaltered or down-regulated in PASS2 and PAO1. T6SS was reported to be active in CF patient infections (197), however, specific reasons for activation during hypoxic stress need to be further investigated.

6.3.9 Other proteins differentially expressed under hypoxic stress:

Among the few proteins that were differentially abundant in all strains, OprG was highly elevated in abundance in all strains, in excess of 5-fold in PASS2. OprG, an outer membrane protein was reported anaerobically induced and a contributor to the bacterial cytotoxicity towards a human bronchial epithelial cell line (244). Another outer membrane porin, OprE was increased in abundance in PAO1 and PASS2 but not significantly altered in PASS3. OprE was noted to be induced specifically under anaerobic growth in PAO1. However, transport functions of OprE remains unknown (245). The CupA fimbrae (PA2129-PA2132) proteins assist *P. aeruginosa* in biofilm formation (246). CupA2-A5 proteins were exclusively upregulated in PASS3 (3.2-4.6 fold) and unchanged in PAO1 and PASS2. This observation is in agreement with the previous report by Vallet-Gely et. al (247) and Alvarez-Ortega *et al* (128) that it is induced under hypoxic conditions. Lastly, increased abundance of ribosomal proteins were observed in all strains during hypoxic growth. The expression of 50S ribosomal protein L31 (PA5049) was dramatically higher, with $\log_2$ fold changes up to +6 measured in PAO1 in both SWATH and TMT technique.

6.4 Discussion:

It is becoming increasingly evident that the adaptation of *P. aeruginosa* to hypoxic conditions, expected within mucus plugs of CF patients, increases biofilm formation (123, 248), alginate production (116), antibiotic resistance (249) and production of virulence factors (124, 125) thus profoundly contributing to the establishment of persistent chronic infection in the CF lungs. To decipher the network of proteins that underpin the adaptation of *P. aeruginosa* to the hypoxic condition, we generated extensive protein expression profiles of two *P. aeruginosa* CF isolates (PASS2 and PASS3) and a laboratory strain PAO1 (ATCC 15692), using TMT and SWATH-MS approaches. Several previously conducted studies examined the response of *P. aeruginosa* to oxygen limitation stress using transcriptomics (128, 212, 214, 215) proteomics (212, 213, 250) and phenotype studies, predominately using the PAO1 strain. Hence, one of the key aims of the study was to assess if there are different adaptations to the hypoxic stress in CF isolates compared to the laboratory adapted

strain PAO1. To trigger expression of *P. aeruginosa* proteins that typically occur in CF lungs, we attempted to simulate the CF lung environment through a combination of (I) oxygen restriction (<1%) (II) growth medium (SCFM); with nutrient composition mimicking CF lungs (141) (III) and a pH of ~ 6.8 similar to airway surface liquid (ASL) in CF patients (141).

Compared to previous studies that attempted to understand molecular mechanism of adaptation of *P. aeruginosa* PAO1 to low-abundance of oxygen (128, 212, 214, 215), our study, with quantification of 3,967 proteins, represents the most comprehensive proteome coverage of clinically relevant strains of *P. aeruginosa*, providing an opportunity to understand expression dynamics of proteins which were previous not detected. Notably, a diverse class of proteins were differentially expressed in PAO1, PASS2-3 on exposure to hypoxic conditions. One of the striking observations was concurrent up-regulation of aerobic and anaerobic respiration proteins on exposure to hypoxic stress compared to normoxic baseline (Table-6.2 and 6.3). Although previous reports (128, 181) through transcriptomic and culture based studies showed similar observation in *P. aeruginosa* laboratory strains (PAO1, ATCC 9021), our study for the first time showed protein based evidence for concurrent up-regulation of such proteins in CF isolates. Of specific interest, were *cbb₃* oxidases (*cbb₃*-1 and *cbb₃*-2) found up-regulated in all strains. Recently Arai *et.al* (234) concluded that, of five oxidases in *P. aeruginosa*, *cbb₃*-1 and *cbb₃*-2 possess equal and highest affinity to the oxygen (K_m value of ~0.25μM) which is one magnitude higher compared to the other three terminal oxidases (K_m value of ~4 μM) (234). Notably, both *cbb₃*-1 and *cbb₃*-2 have a high sequence similarity of 87% (PAO1, catalytic subunit) but *cbb₃*-1 is constitutively expressed under both aerobic and anaerobic conditions, whereas, *cbb₃*-2 is explicitly expressed under microaerobic conditions (234). Consistent with this observation, in response to hypoxic stress, subunits of *cbb₃*-2 enzyme of strain PASS2 showed a higher magnitude of expression compared to *cbb₃*-1.

Another noted advantage of *cbb₃*-1 oxidase is protection from reactive oxygen species, a stress often faced by *P. aeruginosa* in CF lungs (234). Of note, *P. aeruginosa* PAO1 *cbb₃-1* and *cbb₃-2* double mutant failed to grow under planktonic conditions in

SCFM (0.4% O_2) in the absence of KNO_3. Additionally, *P. aeruginosa* PAO1 *cbb₃*-1 and *cbb₃*-2 double mutant grown in SCFM formed thinner biofilms compared to the wild-type strain underscoring its central role not only in respiration but also in pathogenesis. Furthermore, the *cbb₃* oxidases are unique compared to other heme-copper oxidases in the sense that their active site structurally resembles with that of nitric oxide reductases (NORs) evident by NOR activity of purified *Pseudomonas stutzeri cbb₃* oxidases (178).Lastly, *P. aeruginosa* PAO1, *cbb₃* oxidases were shown to alter anaerobic growth, the denitrification process, and cell morphology under anaerobic conditions (251).

These observations made in this study, along with previous reports (128) conclude that the induction of *cbb₃* oxidases significantly contributes to the respiration of *P. aeruginosa* in hypoxic and anaerobic environments by efficiently harnessing scantly available oxygen and through contributing to the pathophysiology. Hence, up-regulation of *cbb₃* oxidase, specifically, *cbb₃*-2 might potentially serve as a surrogate marker for metabolic adaptation of *P. aeruginosa* to the hypoxic conditions (214).Along with proteins in relation to aerobic respiration, this study showed simultaneous overexpression of several nitrate respiration pathway proteins, in all strains. Strain PASS2 displayed a high elevation of such proteins, whereas PAO1 and PASS3 generally showed lower levels or no induction (Table-6.2). We previously reported (138), PASS2 as a unique strain (among PASS1-4 and PAO1) due to reduced motility, loss of *psl* gene cluster resulting in reduced biofilm which are typical signs of chronic stage infection adaptability to the host.

Co-expression of anaerobic and aerobic pathway proteins has been previously shown by two independent studies (128, 181). Alvarez-Ortega et.al (128) through transcriptomic profiling of *P. aeruginosa* grown in SCFM under microaerobic (2% and 0.4% oxygen) and anaerobic conditions showed an overlapping set of genes expressed between two conditions and concluded that *P. aeruginosa* responds to oxygen limitation along a continuum (128). Chen and colleagues (181) showed, *P. aeruginosa* performs simultaneous oxygen and nitrogen respiration under low dissolved oxygen concentration (0–2.2 mg/L) (181). Notably, antibodies for

denitrification enzymes, NarG and NapA were detected in the sera of CF patients, underscoring their importance in the survival of *P. aeruginosa* in CF host (122). Additionally, CF airway surface liquid and sputum were reported to have ~400μM of nitrate (2, 15), sufficient to support the growth of *P. aeruginosa*, during hypoxic conditions. SCFM used in this study was supplemented with similar concentrations to mimic the anaerobic growth environment of *P. aeruginosa* strains *in vitro*. In a previous study, SCFM devoid of KNO_3 could not support the growth of *P. aeruginosa* PAO1 under strict anaerobic conditions (128), indicating the indispensable role of respiration through denitrification in the survival of *P. aeruginosa*.

Noticeably, a higher magnitude of expression of *cbb3* oxidases and denitrification enzymes in PASS2-3 compared to PAO1 may indicate that CF isolates, compared to laboratory strain PAO1, are better equipped with mechanisms for survival under hypoxic conditions. However, confirmation of the finding in a larger cohort of CF isolate may be necessary.

Of note, evolutionarily, *P. aeruginosa* has one of the highly branched respiratory chains, which is especially useful for the rapid acclimatisation into a challenging niche including CF lungs (6). Additionally, differentially regulated expression of such respiratory enzymes aids in enhancing the capability of the bacteria to respond to stress including hypoxia. Hence, up-regulation of *Cbb3* oxidases, specifically in higher magnitude in PASS strains may be one of the contributing factors among others, for the long-term persistent colonisation *P. aeruginosa* in the CF lungs.

It is worth mentioning that, *Cbb3* oxidases are almost exclusively present in Proteobacteria and the enzymatic machinery for survival in anaerobic conditions is characteristic of few bacteria including *P. aeruginosa* and absent in humans. Considering the emergence of multi- and pan-antibiotic resistance strains of *P. aeruginosa*, proteins for instance, *cbb3*-2 oxidase may potentially serve as a novel and viable drug target, which may help to reduce the pathogen burden on CF patients. However, efficacy of such proteins as drug targets warrants further investigation.

Interestingly, CF isolate, PASS2, on exposure to hypoxic condition showed an increased abundance of a subunit of respiratory cyanide-insensitive quinol oxidase, CIO-A, compared to the aerobically grown counterpart. Cyanide-insensitive quinol oxidase (CIO), is essential in self-protection from autogenic virulence factor, hydrogen cyanide. Hence, increased abundance of the CIO enzyme may likely provide a selective advantage to the bacterium in the cyanogenic environment such as CF lungs with typically measured hydrogen cyanide concentration of up to 150μM (87, 252). Furthermore, considering up-regulation of CIO-A in PASS2 but not in PASS3 and PAO1, as a future experiment, it would be interesting to determine whether PASS2 synthesises more HCN compared to PAO1 and PASS3.

It was interesting to note that the exposure to the hypoxic stress triggered increased expression of several virulence determinants and other proteins that may help *P. aeruginosa* in the pathogenesis. For instance, then majority of components of T6SS were up-regulated in PASS3 and, OprG was found up-regulated in all the strains upon exposure to hypoxic stress. Both, T6SS and OprG have been indicated as important for *P. aeruginosa,* in causing damage to the host and out-competing the co-existing pathogens (197, 199, 244). Thus, exposure of *P. aeruginosa* to the reduced oxygen condition may result in relatively more virulent phenotype, causing more damage to the host environment.

P. aeruginosa requires iron (at least 10^{-6} M) as an indispensable element for cellular functions (253). *P. aeruginosa* has dedicated pathways for the acquisition of ferric (Fe^{3+}) and ferrous (Fe^{2+}) forms of iron (239). The ferric form of iron is predominantly acquired through heme and nonheme components (such as siderophores) and the ferrous form is transported via the Feo system using redox-cycling phenazines (237). Typically, ferric iron bound complexes bind to TonB-dependent outer membrane receptors (239), and internalised to the periplasmic space using electrochemical proton motive force (PMF) as a source of energy (254). A periplasmic binding protein binds with the iron-complex and assists the transport via the cytoplasmic membrane, finally delivering the iron to the cytoplasm (239).

The concentration of iron in the lungs of healthy individuals is usually very low and relatively inaccessible for the invading pathogens (145). On the contrary, recent studies showed unexpectedly high concentrations of extracellular iron in the respiratory tract secretions of CF patients (242+/-47 ng/mg (144, 145)). However, notably, the majority of iron in the CF sputum is bound to the host or bacterial chelators (example: ferritin) and is biologically inaccessible. Compared to the CF sputum, SCFM has much higher biologically accessible levels of iron (3.6 mM (141, 222)).

Intriguingly, on exposure to hypoxic growth condition, multiple proteins involved in both the ferric and ferrous iron acquisition by *P. aeruginosa* were decreased in abundance, largely in PAO1 (Table-6.4), in comparison to normoxic baseline. Specifically, proteins involved in the biosynthesis of pyoverdine and pyochelin, ferrisiderophore and heme receptors, Ton-B dependent receptors, and ferrous iron transports were decreased in abundance in PAO1. Additionally, down-regulation of the heme-based iron acquisition system in PAO1 upon oxygen restriction was observed. Lastly, a probable ferrous iron transport protein FeoB, was decreased in abundance in PAO1, indicating an overall decrease in the abundance of the iron metabolism and acquisition pathway proteins.

It has been reported that the *P. aeruginosa* intracellular iron level could be affected by oxidative stress (255-257). Through a genome-wide transcriptome analysis, Chang *et al* (255) demonstrated repression of multiple genes (e.g. *fpvA, pchA-F, fptA, pvdS*) involved in iron metabolism in *P. aeruginosa* PAO1, when grown in LB under oxidative (H_2O_2) stress (257). Notably, our study along with previous reports (128) demonstrated up-regulation of proteins required for an oxidative stress response in *P. aeruginosa* (Table-6.5). For instance, SodB (PA4366), KatA (PA4236) and CcpR (PA4587) (Supplementary Figure-9.11), proteins required for resistance against oxidative stress in *P. aeruginosa* were up-regulated in all strains. Moreover, although the majority of iron metabolism proteins differed in expression with that reported in Hare *et al* (257), the study showed down-regulation of HemO (PA0672) in *P. aeruginosa* PAO1 exposed to oxidative stress (257), which is consistent with the

findings in this study. One of the likely explanations for the link between hypoxic and oxidative stress through superoxide generation is, during hypoxic condition, electron transport in the electron transport chain slows, increasing the reduction state of electron carriers. This aggregation of reducing equivalents in turn favours production of superoxide at low oxygen concentrations by elevating the electrical potential for reduction of oxygen by a single electron to superoxide (258, 259), ultimately causing oxidative stress to the bacterium.

Another interpretation of down-regulation of proteins involved in ferric iron metabolism is, ferric iron (Fe^{3+}) intake through heme and siderophore-mediated pathways. For instance, Ton-B receptors mediated intake, is energised by PMF (253, 254). Hence, through down-regulation of iron (Fe^{3+}) intake which requires PMF, *P. aeruginosa* might deviate the PMF for driving energy generation through ATP synthesis.

Also, consistent with our observation of decreased abundance of TonB-dependent receptors and ferripyoverdine receptors in PASS2 and PASS3, CF isolates of *P. aeruginosa* lacking type I ferripyoveridine receptor, *fpvB* and several other TonB-dependent receptors were isolated from CF patients (260).Overall, the results demonstrate a reduction in iron acquisition in *P. aeruginosa* PAO1 in connection with increased expression of oxidative stress related proteins. This is consistent with the previous reports (257) (255), which through transcriptomic analysis of *P. aeruginosa* PAO1 exposed to oxidative stress reported, a down-regulation of iron metabolism proteins. However, it is unclear whether such modulations in a long-term lab adapted strain such as PAO1, provide any metabolic advantage.

One of the aims of this study was to investigate if PAO1 and PASS strains have similar adaptations under hypoxic stress. Interestingly, several proteins were uniquely differentially expressed in PAO1 and PASS strains (Figure-6.3). For instance, proteins in relation to secretion system were heterogeneously expressed in PAO1 and PASS strains when exposed to hypoxia. T1SS was down-regulated in PAO1 and generally not significantly altered in PASS strains. One interpretation of

this finding is, T1SS, apart from secreting virulence factors, also secrets iron scavenging, hemophore protein HasAp (262). This is consistent with down-regulation of iron acquisition pathway proteins, as explained in the previous section. On the other hand, T6SS components were up-regulated almost exclusively in PASS3 and not significantly changed in PAO1 and PASS2. Several stress response proteins (KatA, CcpA) were uniformly elevated in abundance in all strains when exposed to hypoxia, indicating some of the stress response mechanisms are potentially conserved in *P. aeruginosa*.

In summary, our data strongly support the hypothesis that *P. aeruginosa* concurrently induces aerobic and anaerobic respiration pathways along with fermentation as a response to oxygen restriction rather than having the discrete machinery to tackle anaerobiosis. Most importantly, PASS strains compared to PAO1, may have much more robust mechanisms to deal with oxygen restriction stress. All investigated strains of *P. aeruginosa* display a uniform response to hypoxia related oxidative stress. It was illustrated that hypoxia in association with the resultant oxidative stress altered iron metabolism, by down-regulating the majority of proteins in the pathway. Furthermore, we observed a considerable diversity in the expression profiles of several proteins in PASS strains compared to PAO1 under hypoxic stress indicating the absence of a "one size fits all" mechanism in *P. aeruginosa* strains to cope with external stress such as hypoxia. Our data also showed the exposure of *P. aeruginosa* to the hypoxic condition, triggered several stress response, virulence and adaptive features which assist the bacteria in damaging the host and outcompeting the co-existing pathogens enabling the establishment of stable, long-term persisting colonies. Hence, reduction of hypoxic regions in CF lungs would be unfavourable to *P. aeruginosa* colonization and would probably improve the patient health. In conclusion, with an approach of comprehensive mass spectrometry-driven characterisation, our study has provided a wealth of information to further comprehend the "pathoadapation" of *P. aeruginosa* in CF lungs.

Chapter-7

General Discussion

7.1 <u>Overview</u>

The principle aim of this study was to investigate the role of *P. aeruginosa* cellular proteins in adaptation to the highly compartmentalised, harsh microenvironment encountered in CF patients' lungs. In this context, an extensive whole cell global proteome and membrane proteome analysis of four novel *P. aeruginosa* CF sputum isolates (PASS1-4) was performed by LC-MS/MS. To mimic the CF lung environment, a defined, synthetic CF sputum medium (SCFM), which recapitulates the CF lung nutrient and pH conditions (141) was used. The impact of oxygen restriction in SCFM was also studied. As much of the *P. aeruginosa* literature has been developed based on the PAO1 reference strain, this was used as a comparator to elucidate CF lung-specific molecular adaptations used by PASS strains.

The comparative proteome analysis lead to the identification of several proteins in PASS strains which may potentially provide selective and survival advantage to *P. aeruginosa* during persistent infections in the CF lungs. Of note, there was considerable heterogeneity amongst the proteomes of PASS strains which were different to that of PAO1, indicating a variety in adaptation strategies that are possible by CF isolates. Additionally, the proteome analyses conducted here emphasise that the common PAO1 lab strain is relatively a poor model to understand *in vivo* molecular adaptations undertaken by *P. aeruginosa* in CF lungs which are able to benefit from lateral gene transfer and advantageous mutation.

Greater understanding of *P. aeruginosa* in the CF lung is of huge importance as the occurrence of multi-drug resistant strains is continuously emerging, with treatment options under pressure. Therefore, this thesis contributes by detailing comprehensive proteome analyses of *P. aeruginosa* CF isolates to discover proteins that are advantageous for pathogen survival, and may represent useful pathways or targets for new drug development.

7.2 Essential use of CF isolates of _P. aeruginosa_ to understand host adaptation.

The majority of the "omics" studies used to understand the molecular adaptations of _P. aeruginosa_ in CF lungs have been conducted using the laboratory-adapted PAO1 strain (128, 212, 213, 250, 263), with the exception of a few recent studies (151, 173, 264) which used the Australian epidemic strain 1, (AES-1), a transmissible CF isolate, (shares 73-78% genome similarity with PASS1-4 (Table-9.6)). PAO1 was originally obtained from a burn wound in 1955 (216), since then, it has been passaged in laboratory media and used worldwide as a common _P. aeruginosa_ reference strain. Although there is considerable genomic overlap between PAO1 and other _P. aeruginosa_ strains, there are also unique genomic pathogenicity islands and deletions as reported by us (138) and others (265-267) which aid _P. aeruginosa_ in surviving in the harsh conditions experienced in chronic CF lungs infections (Figure-7.1). For instance, partial or complete loss of several genes involved in the biosynthesis of siderophore, pyoverdine were observed in PASS2 and PASS4 (138), resulting in no detectable traces of fluorescent pyoverdine. There are several reports showing "social cheater" strains (268) of _P. aeruginosa_ lacking siderophore production and such strains may rely on siderophores produced by other bacteria in the polymicrobial niche of CF patient lungs. Additionally, on exposure to oxygen restriction, decreased abundance of several proteins involved in biosynthesis of siderophore, pyochelin, compared to normoxic baseline, was observed in PASS3 when grown in SCFM (Table-6.4, Chapter-6). Loss of such useful features such as siderophore biosynthesis, in CF lung adapted _P. aeruginosa_ might have been due to factors including; (i) ample availability of iron in the CF sputum (269) (ii) preference of heme and citrate bound iron which is available in abundance in CF sputum (iii) low oxygen concentration in CF sputum which leads to increased concentration of readily available, soluble ferrous iron (270).
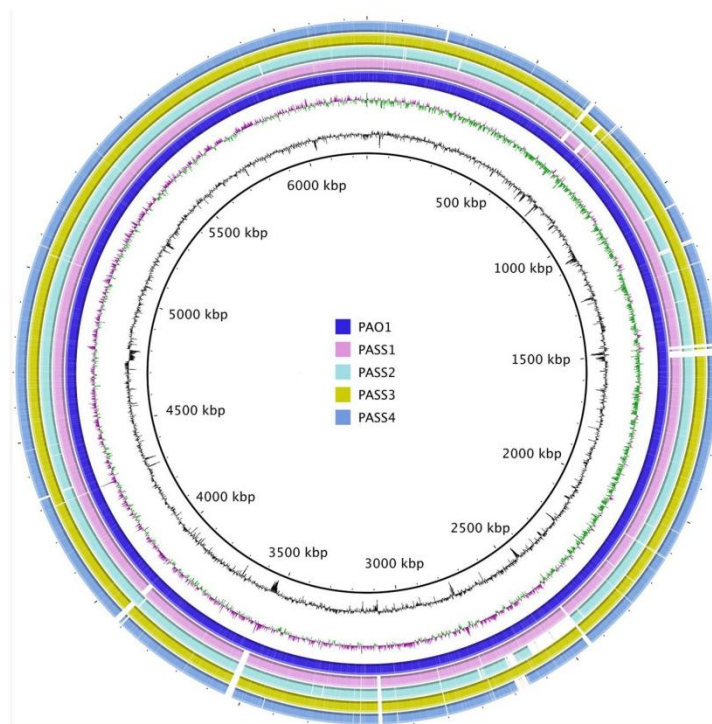
Figure-7.1: Circular representation of *P. aeruginosa* PASS1-4 draft genomes aligned against the reference genome of PAO1.The two innermost circles represent the GC content and the GC Skew respectively. Figure adopted from Penesyan *et al* (138)**.**

Hence, it is rationalised that studying low passaged *P. aeruginosa* CF isolates grown in specialised laboratory medium such as SCFM, till exponential phase, will be more informative in providing a closer *in vitro* picture of the biomolecular features enabling this pathogen to colonise the CF lung. Of note, although it is likely that the stationary growth phase is a better representative of the growth characteristics of *P. aeruginosa* in CF lungs, the mid-logarithmic phase was predominantly chosen for examination in this study (Chapter-5 and chapter-6). We rationalised that, in the stationary phase, cells are latent in nature, on the contrary, exponential phase represents most active phase of growth of the bacterium. Hence, it is likely that, most of the genes are activated and expressed in this phase, enabling identification and quantification of such proteins via mass spectrometry, leading to wider proteome coverage.

The PASS strains reported in this thesis were isolated from CF patients of diverse age, gender, antibiotic treatment and CFTR mutation groups, hence, they provide a broad sampling of molecular features associated with *P. aeruginosa* in CF lungs (section 1.4.6).

7.3 SCFM medium mimics CF lung nutrient conditions

Metabolic versatility of a pathogen could be in part demonstrated by the range of carbon sources and other micronutrients utilised for survival. A hallmark of the CF airway surface is severe dehydration and unusual nutrients which are often absent in healthy human lungs including amino acids, DNA, mucins, sugars, phospholipids, iron, nitrite, etc. (143). These nutrients are an excellent growth medium and known to support high densities of *P. aeruginosa* >10^8 CFU/mL (271). The nutrients are known to influence several clinically relevant phenotypes of *P. aeruginosa* including, cell-cell communication, biofilm formation and surface motility (141). Palmer *et al* generated a CF sputum nutritional profile and utilised the knowledge to create a defined synthetic medium, termed SCFM. They found striking similarities in carbon substrate preferences, growth pattern and gene expression profiles of *P. aeruginosa* when grown in SCFM and crude CF sputum, indicating SCFM closely simulated nutrient conditions typical of the CF lung.

For the proteomic experiments of *P. aeruginosa* in this study, SCFM was predominantly used and the growth conditions were contrasted with commonly used laboratory media including M9-glucose minimal medium and LB broth. Growth in SCFM triggered expression of several proteins which were generally not induced or not significantly alerted in expression when grown in M9-glucose (222). For instance, OprQ porin and MexB (PA0426) involved in antibiotic resistance and outer membrane chaperon SurA (PA0594) were differentially expressed in PASS strains compared to PAO1, when grown in SCFM and not in M9-Glucose growth condition (222), underscoring the importance of growth medium in mimicking CF lung like conditions *in vitro*.

It is important to note that, although SCFM is rich in several key nutrients, similar to CF sputum (141), it may not completely represent all the biomolecules present in CF respiratory tract. For instance, one such biomolecule is glycoprotein mucin. The epithelial cell surface of CF patient's respiratory tract are typically covered with thick, dehydrated mucus, mainly comprised of mucins. Such mucus deposits

contribute significantly to the disease pathophysiology through formation of a physical shield for the pathogens resulting in hypoxic environments. Consequently, *P. aeruginosa* migrates to these mucus zones and respond with the production of alginate and hydrogen cyanide, thus deteriorating the lung environment (116, 272).

7.4 Proteins that assist microevolution/adaptation of *P. aeruginosa* during chronic infection:

Genetic analysis has revealed the initial colonisation of *P. aeruginosa* happens through unidentified environmental reservoirs and patient to patient transmission (11). Following the acquisition, *P. aeruginosa* employs a multifactorial and complex system to establish infection, evade host immune system and adapt to the lung micro-environment. Several longitudinal (140, 273, 274) studies have reported numerous adaptations strategies employed by *P. aeruginosa* using phenotypic assays and genetic techniques, in acute and chronic phase of infection.

In the following section, the most relevant changes observed in the proteomes of PASS strains and PAO1 found in this thesis will be discussed in the context of their possible biological roles in CF pathophysiology.

7.4.1 Hoard no more: Loss of virulence, motility as an indicator for adaptation to chronic CF lung infection:

Membrane subproteome analysis of PASS1-3 and PAO1 (Chapter 5) lead to the collective identification of over 990 membrane proteins, thus the study represents the most comprehensive quantitative membrane subproteome of clinically relevant *P. aeruginosa* till date (222). Strikingly, the study revealed a significant reduction in the abundance of many proteins in PASS1-3 compared to PAO1, which are essential for chemotaxis, aerotaxis, motility (swarming, twitching), adhesion and host immune system evasion.

Consistent with the reduced expression of motility and adhesion appendages, PASS1-3 strains and selective flagella and pili gene mutants of *P. aeruginosa* PA14 demonstrated reduced adhesion to CF sputum *in vitro* (222). Additionally, PASS2 and PASS3 showed decreased *in vitro* binding to purified porcine gastric mucin and

concomitantly both PASS2 and PASS3 formed poor biofilms in the flow-cell (138). Furthermore, along with flagella and pili, Psl polysaccharide and lectins are known to assist *P. aeruginosa* adhesion to the host epithelial cell surfaces (140, 275). PASS2 strain has a deletion in the set of genes that encode the Psl polysaccharides and on the other hand, PASS1-3, showed deletion of the *lecA* gene (138), which further supports the view that PASS strains portray reduced adhesion to CF sputum and purified mucins. These factors might be a significant contributor towards the reduced cytotoxicity of PASS2 and PASS3 against *C. elegans* as previously demonstrated by us (138).

Despite these findings shown in Chapter 5, previous work by others suggested that the loss of flagella *per se* may not confer immune system evasion capacity, but rather the loss of motility provides this protection (103). Interestingly, in a semi-solid agarose plate motility assay, PASS2 and PASS3 showed significantly less motility compared to PAO1, confirming the proteome level down-regulation in a phenotype of reduced motility.

Nonetheless, it was interesting to note that, although proteins in relations to motility and adhesion, were decreased in abundance in almost similar magnitude in both PASS2 and PASS3 compared to PAO1, when grown on solid agarose plates, only PASS2 exhibited little or no motility, while PASS3 showed reduced motility which was higher than PASS2. This may implicate that in the absence or reduced expression of flagella and pili, there are other factors that contribute to the motility of the bacterium. Consistent with our observation, Köhler and colleagues demonstrated that, *P. aeruginosa* PAO1 FliC mutant showed decreased but not abolished swarming motility, confirming the role of non-flagella proteins in the motility of the bacterium (276). Furthermore, PAO1 double mutants expressing neither flagella nor type IV pili (FliC, PilA) was surprisingly shown to display sliding motility mediated in part through the surfactant, rhamnolipids (277). Rhamnolipid is a bacterial surface wetting agent, absence of which might cause reduced virulence and motility. Hence, although expression of flagella and pili proteins is down-regulated in PASS3, other factors including rhamnolipids may assist the bacteria in sliding motility. As a future

experiment, it will be interesting to investigate physical and biochemical properties of surface appendages of PASS2 and PASS3 using microscopy and rhamnolipid detection assay. The results may provide clues about factors that influence PASS3 motility despite down-regulation of proteins that form flagella and pili. Put together, our results indicate, *P. aeruginosa* motility is multi-factorial, however understanding the underlying mechanism of motility warrants further investigation.

Previous studies utilising phenotypic assessment and genetic studies have demonstrated the reduction of motility and evasion of the immune system is a feature of chronically adapted *P. aeruginosa* (103, 275, 278). Thus, reduced motility as a feature of CF isolates found in this study is consistent with these reports and could be useful for the distinction of acute and chronic phase adapted *P. aeruginosa*.

Partial or complete loss of chemotaxis, flagella and pili might come at a cost of motility, however, this attribute appears not to be restrictive for *P. aeruginosa* in CF lungs where the bacterial cells often reside in stagnant mucus plugs and biofilms. According to PaxDb (protein abundance across organisms database) (279), which ranks protein expression according to its abundance in the entire proteome of the organism, flagella and pili proteins are usually of the greatest abundance in *P. aeruginosa*. For instance, FliC protein which makes the monomers of flagella is the most abundant protein (http://pax-db.org/protein/3586353), and type 4 fimbrial proteins, PilA, is ranked ninth most abundant (http://pax-db.org/protein/3589786) in the PAO1 proteome. Hence, synthesis of such highly abundant proteins would be a significant "metabolic burden", so the limited expression of these proteins may enable metabolic "savings" to be reinvested elsewhere. Another significant advantage of reduction of motility proteins is more effective host immune system evasion (57, 58, 280). In recent years, the immunostimulatory nature of flagella has gained increased interest which is evident by its potential use as a vaccine candidate (281).

In summary, loss of motility and adherence appendages is a feature of chronically adapted strains of *P. aeruginosa* and this contributes to persistent infection in CF lungs.

Comparative global proteome analysis revealed decreased abundance of several other virulence-associated proteins in PASS strains compared to PAO1. HcnA and HcnB proteins were down-regulated in PASS1-3 compared to PAO1 when grown aerobically in LB medium. Reduction of hydrogen cyanide (HCN) synthesis might be one of the contributors to the reduced cytotoxicity of PASS strains, towards *C. elegans* (138), as shown by Gallagher *et al.* that HCN caused paralytic killing in a *C. elegans* infection model (191). Consistent with this, *P. aeruginosa* mutants of *hcnABC* genes showed significantly reduced virulence in *Drosophila melanogaster* than the parental cyanogenic strain (282).

One of the interesting observations made by comparing the proteome of PASS1-4 strains with PAO1 grown in LB (Chapter-4) was down-regulation of phenazine biosynthesis proteins (PA4210-PA4216) in PASS2-3 and up-regulation in PASS1 compared to PAO1 (Chapter-3). These proteomic results reflected the findings of phenotypic assays (138), that PASS2-3 did not produce detectable levels of phenazines as measured by UHPLC. Reduced production of the potent virulence factor, phenazine might be one of the contributing factors for reduced cytotoxicity of PASS2-3 strains to *C. elegans* (138). Consistent with this observation, *P. aeruginosa* mutant strains lacking phenazine production, specifically pyocyanine, have been shown to be attenuated in their ability to infect mouse lungs in an acute pneumonia model of infection, compared to the isogenic wild-type strain (89).Membrane subproteome analysis of PASS strains in comparison to PAO1 revealed down-regulation of Type 1 secretion system (T1SS) proteins (AprD, AprE and AprF) when grown in the M9-gluscose medium. The well-characterized Apr system consists of AprD (ABC transporter), AprE (adaptor), and AprF (OMF) which facilitate the extracellular secretion of virulence factor AprA (283). Hence, it is logical to imply that the reduction in expression T1SS secretion machinery may translate to reduced

secretion of virulence factor AprA, resulting in a reduction of AprA mediated virulence.

Reduced expression of virulence determinants or mutation of virulence related genes is one of the features often associated with long term CF adapted strains of *P. aeruginosa* (69, 284). It is important to note that the patients, from whom we isolated PASS strains, were of age ranging from 23 to 40, indicating the strains may have well adapted for a long term in the CF lung niche. One of the likely explanations for requirement of trait of reduced virulence during long term adaptation is, evasion of host immune system, since immune system recognises several virulence factors and tends to eliminate cells that express such virulence determinants (69, 285). Hence, it might be advantageous to suppress virulence at the cost of gaining a survival advantage.

Interestingly, comparative proteome analysis of PASS1-4 and PAO1 following the growth in LB medium revealed, PAO1 expressed an array of membrane transport proteins involved in the uptake of a broad range of biomolecules including carbohydrates, amino acids, polyamines and other organic nutrients. However, no expression of genes involved in the biosynthesis of such biomolecules was observed. On the contrary, PASS1-4 expressed smaller number of membrane transport proteins and relatively a large number of proteins involved in the biosynthesis of amino acids, carbohydrates, polyamines, nucleosides and nucleotides (138). This indicates, PAO1 being laboratory adapted strain, may transport "ready-made" nutrients from LB, compared to PASS1-4 strains, which may rely on own metabolism to synthesise such essential nutrients. This could mean a longer lag-phase of growth of PASS strains, to allow time for the biosynthesis of nutrients. Intriguingly, we observed a rather similar growth pattern, with no differences in lag-phase and doubling time between PASS1-3 and PAO1 (Figure-9.12).

In agreement with this observation, preliminary phenotype microarray analysis of PASS1-4 compared to PAO1, comparing carbon source utilization, including amino acids, carbohydrates and carboxylic acid indicated that, PASS strains had

significantly reduced ability to utilize those carbon sources compared to PAO1 (unpublished data), which may be due to absence of expression of necessary transporters. However, intriguingly, there were no apparent differences in growth characteristics between PAO1 and PASS strains.

It is worth mentioning that not all virulence factor associated proteins were found down-regulated in our study. For instance, comparison of whole cell proteome of PASS1-4 with PAO1 after growth in LB broth revealed an increased abundance of several proteins of type 6 secretion system (T6SS) in PASS4 compared to PAO1(Chapter-4), while expression levels in PASS1-3 were similar to that of PAO1. Furthermore, on exposure to hypoxic conditions, PASS3 showed an increased abundance of several H1-T6SS and H2-T6SS type of T6SS proteins (PA0074–PA0091) compared to normoxic growth (Chapter-6). Sana and colleagues (18) recently showed that, in *P. aeruginosa*, the H2-T6SS mediates entry into the mammalian host, while, H1-T6SS secretes bacteriolytic effectors and might provide a survival advantage to *P. aeruginosa* in the complex polymicrobial environment of the CF lungs.

In summary, several virulence factor associated proteins are altered in expression and a large proportion of them have reduced protein expression in PASS strains compared to PAO1 (an exception is T6SS which was found upregulated in PASS3 and PASS4). The results are in good agreement with previous reports (140) that show reduced virulence is a hallmark of *P. aeruginosa* isolated from patients with persistent *P. aeruginosa* infections and such a phenotype could act as a marker for identifying well adapted *P. aeruginosa* isolates.

7.5 *P. aeruginosa* aerobic and anaerobic respiration in CF lungs:

Global proteomic analysis of *P. aeruginosa,* PASS2-3 and PAO1 in SCFM using SWATH and TMT-MS, under hypoxic stress allowed quantification of over 3,900 high confident proteins which accounts for up to ~71% of the predicted ORFs of *P. aeruginosa* PAO1 (Chapter-6). The data suggests the applicability of proteomics technologies in comprehensive, near-to-complete survey of bacterial proteomes. Of note, an excellent agreement in quantitative ratios (Pearson correlation~0.96) for

proteins between SWATH and TMT was observed in this study. It is likely that the SPS based MS3 scan used for quantification of TMT reporter ions is one of the contributing factors for the high correlation between SWATH and TMT techniques. This is because SPS in combination with MS3 scan was reported to almost completely eliminate the commonly encountered issue of underestimation of the ratios in TMT-MS (168, 169). Additionally, for the proteolysis of *P. aeruginosa* proteins, a combination of Lys-C and trypsin was employed, increasing the availability of C-terminal lysine residues and N-termini for labelling with amine-reactive TMT reagents (168). Consequently, in the MS2, both b- and y-type fragment ions bear a TMT tag, and a MS3 scan on almost any fragment ion will produce TMT reporter ions.

The data revealed differing expression levels of proteins from PASS strains and PAO1 with exposure to hypoxia (O_2<1%) compared to normoxia (O_2~21%) (Chapter-6). Markedly, proteins involved in the aerobic and anaerobic respiration were increased in abundance in PASS2-3 and PAO1. Of interest, terminal oxidases, specifically, *cbb₃*-1 and *cbb₃*-2 and denitrification enzymes (e.g. Nar G, H, I, J) were concurrently up-regulated in PASS2-3 and PAO1. However, strain PASS2-3 showed a higher magnitude of expression or almost exclusive expression of such proteins, compared to PAO1, which may indicate that the PASS strains are better equipped for coping with oxygen restriction that typically occurs in CF lungs. Concurrent expression of aerobic and anaerobic respiration proteins in *P. aeruginosa* PAO1 on exposure to hypoxic conditions were previously shown by Alvarez-Ortega *et al*. (128) in a transcriptomics study, thus, the results presented here at the protein level in both CF isolates are supportive of Alvarez-Ortega *et al*.'s work.

Recently, Arai and colleagues (234) using mutants of terminal oxidases in *P. aeruginosa* PAO1 concluded, of five respiratory oxidases, *cbb₃*-1 and *cbb₃*-2, have equal and highest oxygen affinity, while *cbb₃*-1 is constituently expressed, *cbb₃*-2 is induced mainly under hypoxic condition. This is consistent with observation made in our study that, of five known oxidases only *cbb₃*-1 and *cbb₃*-2 were found up-regulated on exposure to oxygen restriction, underscoring the indispensible role of *cbb₃* oxidases in

the *P. aeruginosa* respiration during hypoxic condition. Of note, protein NarG was found significantly increased in abundance exclusively in PASS2 and not in PAO1 (Table-6.3, chapter-6). Consistent with this, antibodies for *P. aeruginosa* denitrification enzymes (eg. NarG) were detected in the sera of CF patients indicating it might be one of the highly expressed and important proteins during adaptation of *P. aeruginosa* in CF lungs (122).

Apart from up-regulation of denitrification enzymes, proteins associated with arginine fermentation (ArcDABC) pathway were up-regulated mainly in PASS2-3 and PAO1. Overall, the result suggests the co-existence of aerobic respiration, denitrification and arginine fermentation in PASS strains and PAO1 when grown under hypoxia *in vitro* in SCFM.

On the other hand; compared to PAO1, when aerobically grown in LB, PASS2-4 showed decreased abundance of the majority of proteins involved in *P. aeruginosa* aerobic respiration, including dehydrogenases, ubiquinone biosynthesis proteins, cytochrome bc1 complex, terminal oxidases (*cbb3*-1 and *cbb3*-2 oxidases) and subunits of ATP synthase enzyme. However it needs to be determined if this translates into reduced rate of aerobic respiration in PASS strains compared to PAO1.

To summarize, exposure to hypoxia brought about significant changes in the proteomes of *P. aeruginosa*, PASS strains and PAO1. PASS strains with higher magnitude of expression of *cbb3* oxidases and almost exclusive induction of several denitrification enzymes may have a robust response to hypoxic stress compared to PAO1. Despite debate about whether *P. aeruginosa* grows aerobically or anaerobically in CF lungs, this study shows that PASS2-3 and PAO1 grow well under both normoxic and near anoxic conditions. It is important to note that, CF lungs usually display very heterogeneous oxygen distribution patterns. Hence, the results shown in this study supports the view that, *P. aeruginosa* has the potential for concurrent expression of aerobic and anaerobic respiration proteins, an advantage for survival in CF lungs. The pathogen copes with fluctuating oxygen availability in a continuum rather than activating a singular, discrete pathway for each condition.

Lastly, it is important to note that the *cbb₃* oxidases are phylogenetically most distant to human enzymes and are exclusively present in the bacteria including *P. aeruginosa*, which makes it an ideal conceptual drug target. Given the limited efficacy of present day antibiotic regimens in the treatment of CF airway disease, development of therapeutic interventions targeting hypoxic and/or anoxic metabolism of *P. aeruginosa* might be a promising approach for treatment of chronic CF lung infections (detailed in section 7.7).

7.6 <u>Proteins associated with drug resistance:</u>

P. aeruginosa employs strategies to combat the continuous exposure of antibiotics in CF patient lungs. Comparative analysis of membrane sub-proteomes of PASS1-3 strains with PAO1 revealed several proteins associated with antibiotic resistance and susceptibility (Chapter5) (222), and the majority were increased in abundance in PASS1-3 compared to PAO1. Validation of the results using MICs using the antibiotics from a selection of antibiotics used in CF patient's treatment showed a higher resistance of PASS strains to moxifloxacin, polymyxin and tobramycin compared to PAO1, when performed in SCFM medium (222).

One of the interesting observations was proteins of drug efflux system, MexC, OprJ which are part of MexCD-OprJ multidrug efflux system, found up-regulated in PASS2-3 when compared to PAO1. MexCD-OprJ multidrug efflux systems has been implicated in fluoroquinolones resistance in clinical isolates of *P. aeruginosa* (286). Strikingly, MICs of moxifloxacin, a fourth-generation synthetic fluoroquinolone (a DNA gyrase inhibitor), in PASS2 and PASS3 were two to three-fold higher compared to PAO1 (222). Resistance to moxifloxacin specifically in PASS3 might have been a result of prior *in vivo* exposure to the antibiotic, since, CF patient from whom PASS3 was isolated, was treated with moxifloxacin (222).

To summarize, both quantitative membrane subproteome analysis and MICs of PASS strains (222) suggest that PASS strains are well equipped with over-expressed efflux pumps compared to laboratory strain to combat antibiotics. The regular exposure to the antibiotics in the CF lungs might have contributed significantly to the increased resistance of PASS strains.

7.7 <u>Differentially expressed proteins as potential vaccine/drug targets:</u>

Considering the emergence of multidrug resistance features of *P. aeruginosa*, development of novel drug target is necessary to combat the infection. Additionally, alternative approaches, including the development of vaccines, which do not induce the selection pressure, thus, consequently do not contribute to antimicrobial resistance, could help to reduce infection burden on the patients (287). Despite a large number of patients who succumb with *P. aeruginosa* infections, surprisingly, there is no *P. aeruginosa* vaccine currently available in the market (288).

Unquestionably, proteins that are essential in survival of the bacterium and conserved across multiple pathogens, can be ideal for the development of the broad-spectrum drug targets that can be utilized for treating challenging mixed pathogen infections. In this thesis, compared to laboratory strain PAO1, few proteins were shown to be significantly differentially expressed consistently across all, if not most PASS strains. This may indicate, such features are essential for the survival and necessary for the establishment of more persistent colonies, hence, making them potential drug targets.

One of the widely-studied classes of *P. aeruginosa* proteins as vaccine candidate, and found differentially expressed in our study was flagella and pili proteins. Several studies (281, 288) have validated the use of these proteins and the intact flagella organelle as target antigens for vaccination, due to reasons including (A) high abundance on the bacterial cell surface (B) dynamic immunogenic nature of such proteins (C) phylogenetically distinct nature compared to human proteins and (D) conserved, high occurrence across wide range of pathogenic organisms. For instance, an *in vivo* study using Pneumonia mouse model, Campodonico *et al* showed PAO1

(b-type flagella) and PAK (a-type flagella) strain flagella (including FliD cap protein and basal body components), elicited modest protection against CF isolates of *P. aeruginosa*. Interestingly, they also demonstrated, flagella is more immunogenic compared to monomeric protein building block, flagellin. Arguably, our study (222), including other studies (102, 289) through phenotypic analysis showed, down-regulation of several proteins involved in environment sensing (PA1561, PctA, PctB) and motility (FliK, FlgE, FliD, PilJ) as a notable trait of clinical strains, well established to CF environment, questioning the utility of such proteins for development of vaccine. Additionally, we observed variation in serotype of flagella between PASS strains (138). PASS2 and 4, and PASS 1 and 3, had type-a (45-52 kDa, heterogeneous) and type-b (53 kDa protein) flagella respectively (288), bearing different types of glycosylation, adding to the complexity of the flagella structure. Hence, for CF patients, it will be logical to administrator a multivariate flagella vaccines representing various serotypes, prior to the emergence of the non-flagellated variants of *P. aeruginosa*. Although there have been phase-3 clinical studies, testing flagella vaccines on CF patients (290), no vaccine is currently available for the treatment, thus the field warrants further investigation to establish the role of flagella as a vaccine.

One more class of differentially expressed proteins, potentially a promising drug target includes aerobic (*Cbb$_3$* oxidases) and anaerobic respiration (Dentrification proteins) proteins of *P. aeruginosa*, which were found essential for hypoxic respiration in both clinical and laboratory strains (section 7.5 and 6.3.4). Although, gene knock-out studies were not performed in this thesis to establish the role of *cbb$_3$* oxidases, previous work by Alvarez Ortega (128) demonstrated, double mutant strains of PAO1 lacking terminal oxidases, *cbb$_3$-1* and *cbb$_3$-2*, failed to grow *in vitro*, and form biofilm, under hypoxic condition, indicating the central role terminal oxidases in the survival of the bacterium. Additionally, *Cbb$_3$*-type oxidases are only found in bacteria, often pathogenic ones and specialise in respiration under hypoxic condition, thus making it an ideal drug target to limit the growth of the bacterium. Although structural features of *Cbb$_3$* have been demonstrated in closest related bacteria

Pseudomonas stutzeri (291), there have been no studies till date to understand the utility of protein as a drug target. Hence, considering the central role of the protein in the survival of the bacterium, it will be worthwhile exploring that avenue.

7.8 Limitations of the study:

The *in vitro* planktonic, monoculture, mid-logarithmic growth system employed in this study under rigorous shaking, may not completely replicate the 'real world' growth ecosystem, since the *P. aeruginosa* is often isolated as a complex multispecies community in the biofilm mode in the CF lungs (216, 292). Furthermore, biofilm acts as a physical barrier to the chemicals including drugs, oxidative agents, host immunological molecules and oxygen. As a consequence, it is likely that a variety of genotype and phenotype might have altered when grown under ostensibly similar growth environment, impacting the output of the 'omics' studies. It would be ideal to conduct proteomic analyses from the PASS strains grown in biofilms. Furthermore, considering the recent paradigm technological advancement in the field of transcriptomics and proteomics of the single cell (293, 294), omics analysis of fresh clinical isolates without *in vitro* culturing, may provide an unbiased snapshot of cellular make-up of the pathogens.

Reproducible enrichment of *P. aeruginosa* membrane proteins was one of the key challenges in this thesis (Chapter-5). Using sodium carbonate treatment coupled with ultracentrifugation based enrichment (295), about 31% of membrane protein enrichment was achieved. However, notably, a considerable proportion of cytosolic and uncharacterized proteins were also identified in the membrane protein enriched fractions. Perhaps some of the identified cytosolic or uncharacterized proteins might be interactors of membrane proteins, hence resulting in co-isolation. For instance, in our study, AlgU protein, a cytosolic sigma factor, an important regulator of mucoidy in *P. aeruginosa*, was co-isolated with membrane proteins in all PAO1 and PASS1-3 strains (222). Notably, AlgU protein was previously reported to interact with membrane proteins including MucA and MucB and was shown to behave as a peripheral inner membrane-bound protein (296). So, although AlgU protein is

annotated as a cytosolic protein, there is precedent for it to be co-localized in the membrane, as this study has shown. Hence, large-scale membrane proteome studies could be useful in presenting an opportunity to improve currently annotated proteins.

Achievement of accurate quantification of *P. aeruginosa* membrane proteins across the multiple novel strains with novel protein identifiers, using multiplexing MS techniques was a significant challenge in this thesis. This issue is inherent due to high sequence similarity shared by several proteins of PASS strains and PAO1. For example, protein PilA of PAO1 has 81% sequence similarity with that of PASS1 and PASS3 and 40% with PASS2, which lead to ambiguous quantification. Hence, such proteins were considered "identified but not quantified" and not considered in the final list of differentially expressed proteins. While this issue remains unresolved, it could be an area of focus for future studies to gain more information from proteome studies of novel strains.

One of the challenges in the current implementation of DIA-SWATH-MS is the requirement of a high-quality spectral library needed for peptide identification from multiplexed MS/MS spectra. If the peptide is not registered in the spectral library it cannot be identified and quantified in SWATH-MS. However, recent developments in the field attempt to circumvent this issue (297, 298) by using alternative peptide identification approaches. For instance, a recent approach, DIA-Umpire (297) permits protein identification using conventional database-search from MS/MS data acquired via any currently used DIA method (including SWATH) without the need for a spectral library. Hence it is worthwhile exploring such alternative tools in future studies to gain better coverage of bacterial proteomes.

7.9 Concluding remarks and future directions:

By adopting a systems biology approach, this thesis revealed a number of adaptation strategies used by *P. aeruginosa.* The extraordinary plasticity of *P. aeruginosa* is evident through the diversity of changes in genomes, proteomes and phenotypes as shown within. Such adaptive changes might provide a selective and survival

advantage to *P. aeruginosa* during the micro-evolution in CF patients' lungs. However, future studies will define the aptness of these findings in the development of new therapeutic interventions against *P. aeruginosa*. Importantly, this thesis underscores the need for including multiple *P. aeruginosa* strains pertaining to the diverse clinical background to gain a broader picture of pathoadaptation of *P. aeruginosa*. Wider clinical validation of the proteins of interest as revealed here in additional samples is indispensable and represents the next step in these studies. Furthermore, combining RNA sequencing (RNA-seq) analysis with proteomic analysis might shed more light into gene expression profile of *P. aeruginosa* PASS strains not discovered through a purely proteomics driven analysis. Since RNA-seq information can provide transcriptional features to be located with single-nucleotide precision, comparative trascriptome may provide additional clues about adaptation mechanism of the bacterial strains. Additionally, testing virulence mechanisms of the PASS strains in animal models might provide a more faithful understanding of molecular mechanisms of bacterial adaptation *in vivo.*Despite the current understanding of *P. aeruginosa* pathogenesis, there is still much that we do not understand about the mechanisms that underpin the initial acquisition and transmissibility of *P. aeruginosa* and emerging antibiotic resistance mechanisms employed by the bacteria. Although this study involved comprehensive proteome characterization of four CF isolates of *P. aeruginosa* with diverse characteristics, this does not represent the full diversity of clinical strains that have been reported which would have different attributes in terms of geographical origin, transmissibility, growth, virulence, biofilm formation, and drug resistance (217, 218). Hence, a logical future step to further understand the foundation for chronic infection of *P. aeruginosa* in the CF lung will involve metaproteome characterisation of sequential CF isolates from polymicrobial cultures exposed to sub-lethal level antibiotics, preferably in biofilm mode, since it is the most preferred mode of *P. aeruginosa* growth *in vivo*.

Chapter-8

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Chapter-9

Appendix

9. Supplementary Material:
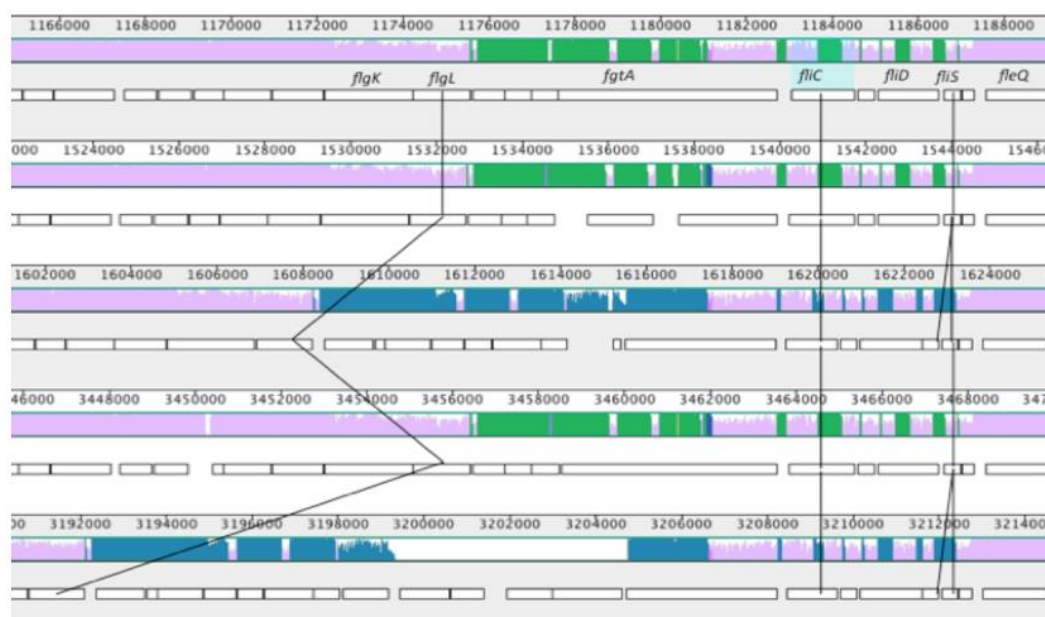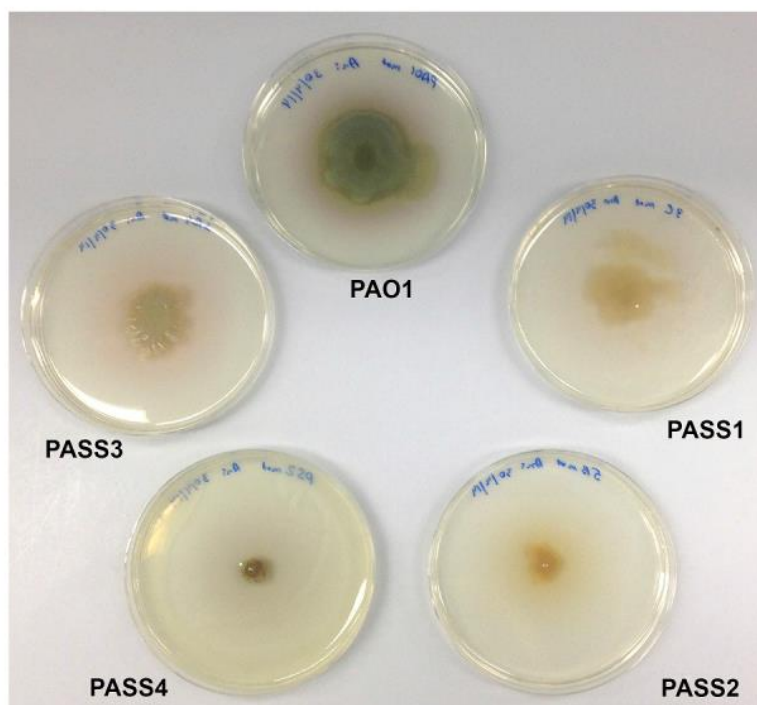
Figure-9.1: Genomic alignment of flagella biogenesis genes in strains PASS1-4 as compared to PAO1 using MAUVE (Source: Supplementary Figure S1 from Peneysan *et.al* (138)) Sequences conserved among all 5 isolates are presented in mauve, sequences shared between isolates PAO1, PASS1 and PASS3 are in green, sequences shared between isolates PASS2 and PASS4 are presented in blue (A). Flagella-mediated swimming motility assay for strains PASS1-4 and PAO1 (B).

Figure-9.2:
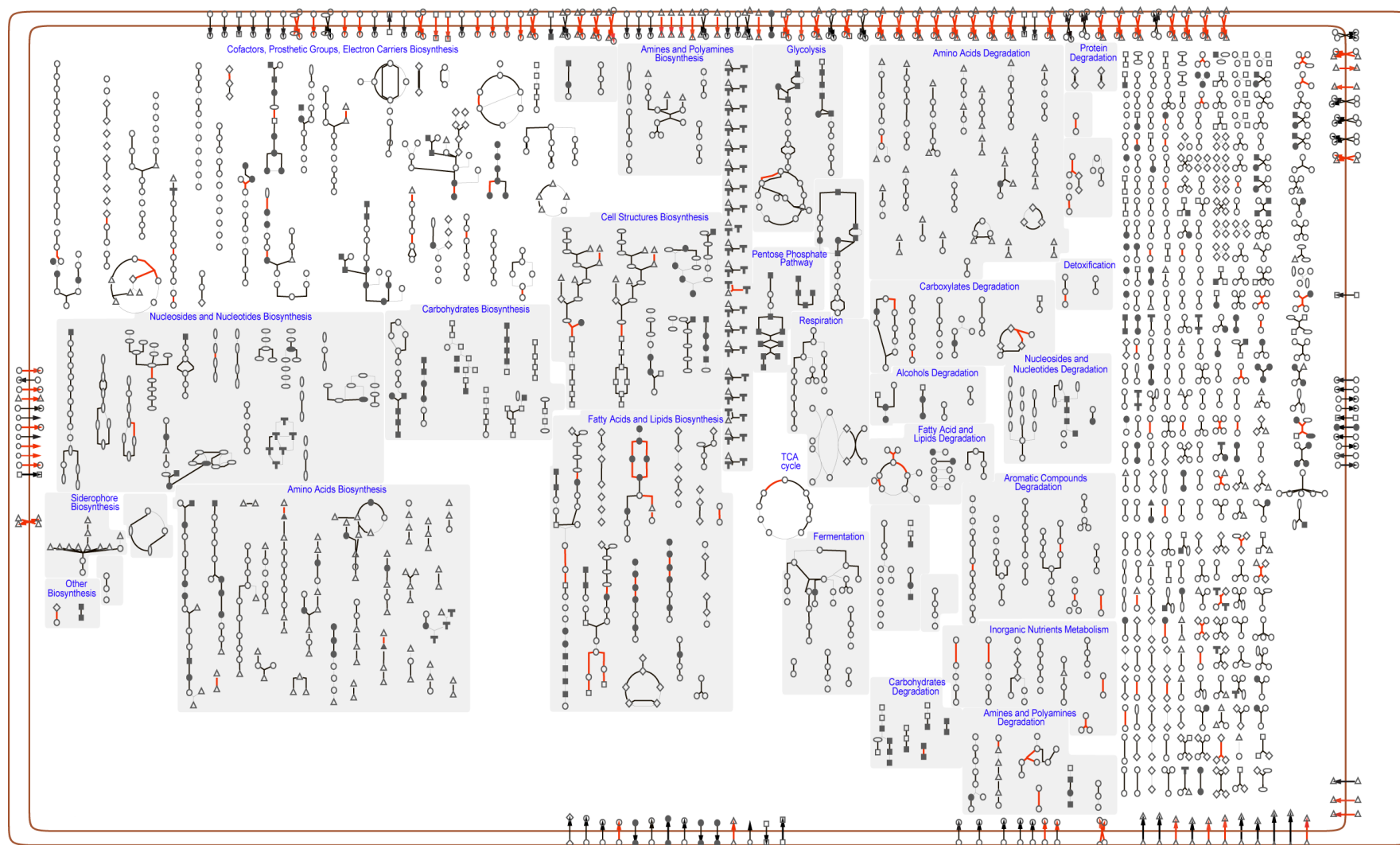
Figure-9.2 (continued from page 199): Pathway Tools 17.5 cellular overview diagram showing proteins/pathways expressed uniquely by PAO1, in red (Source: Supplementary Figure S2 from Peneysan *et.al* (138)): The shapes on the diagram represent specific types of compounds as follows: square—carbohydrate, triangle—amino acid, upside down triangle—cofactor, ellipse (horizontal)—purine, ellipse (vertical)—pyrimidine, diamond—proteins, T-shape—tRNA, circle—all other compounds. The shading of the icon indicates the phosphorylation state of the compound: shaded compounds are phosphorylated; unshaded compounds are unphosphorylated. Shapes/paths located on outer rectangles represent transporters/membrane proteins. The arrows indicate the direction of the transport: pointing in—uptake, pointing out—export.

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Figure-9.3:
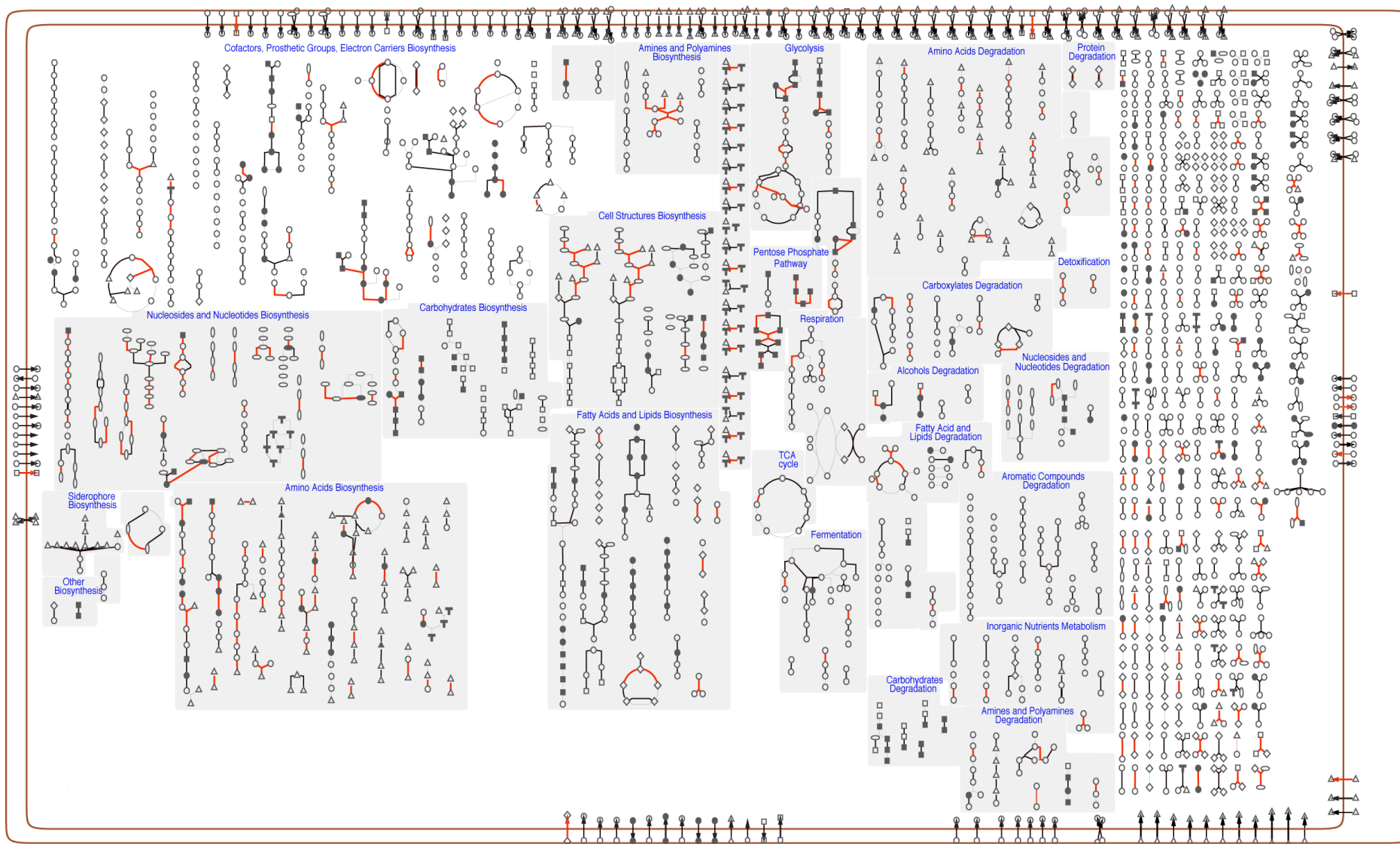
Figure-9.3 (continued from page 201): Pathway Tools 17.5 cellular overview diagram showing proteins/pathways expressed uniquely by PASS1-4 strains, in red (Source: Supplementary Figure S3 from Peneysan *et.al* (138)): The shapes on the diagram represent specific types of compounds as follows: square—carbohydrate, triangle—amino acid, upside down triangle—cofactor, ellipse (horizontal)—purine, ellipse (vertical)—pyrimidine, diamond—proteins, T-shape—tRNA, circle—all other compounds. The shading of the icon indicates the phosphorylation state of the compound: shaded compounds are phosphorylated; unshaded compounds are unphosphorylated. Shapes/paths located on outer rectangles represent transporters/membrane proteins. The arrows indicate the direction of the transport: pointing in—uptake, pointing out—export.

Figure-9.4
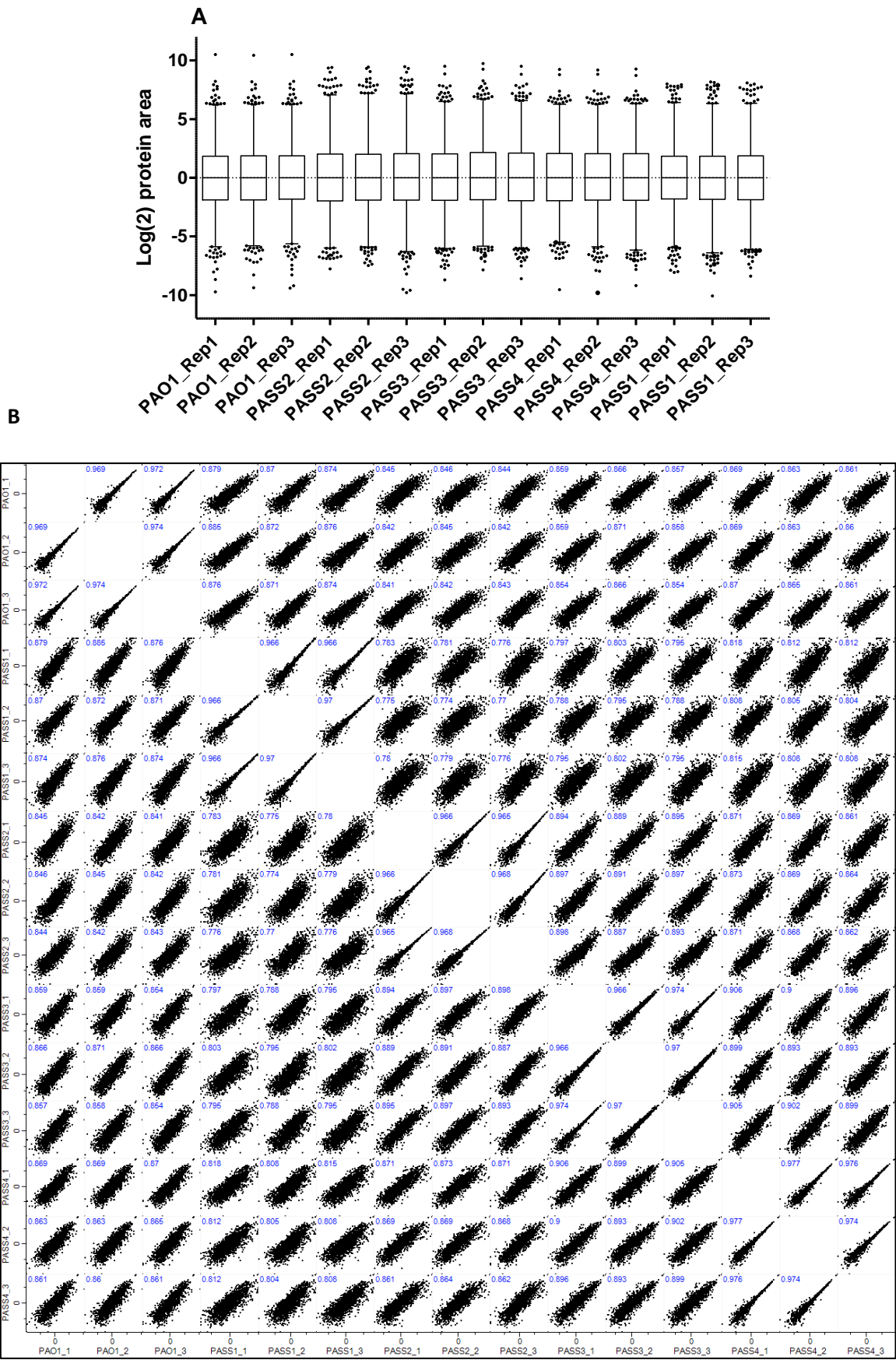
Figure-9.4 (continued from page 203): **Reproducibility of SWATH-MS acquisition:** (A) Box plots of log$_2$ transformed, normalized protein peak areas of individual biological replicates. (B) Multi-scatter plots with Pearson correlation of log transformed protein peak areas of individual biological replicates showing reproducibility between biological replicates. Pearson correlation coefficient close to one represents the higher overlap between replicates.
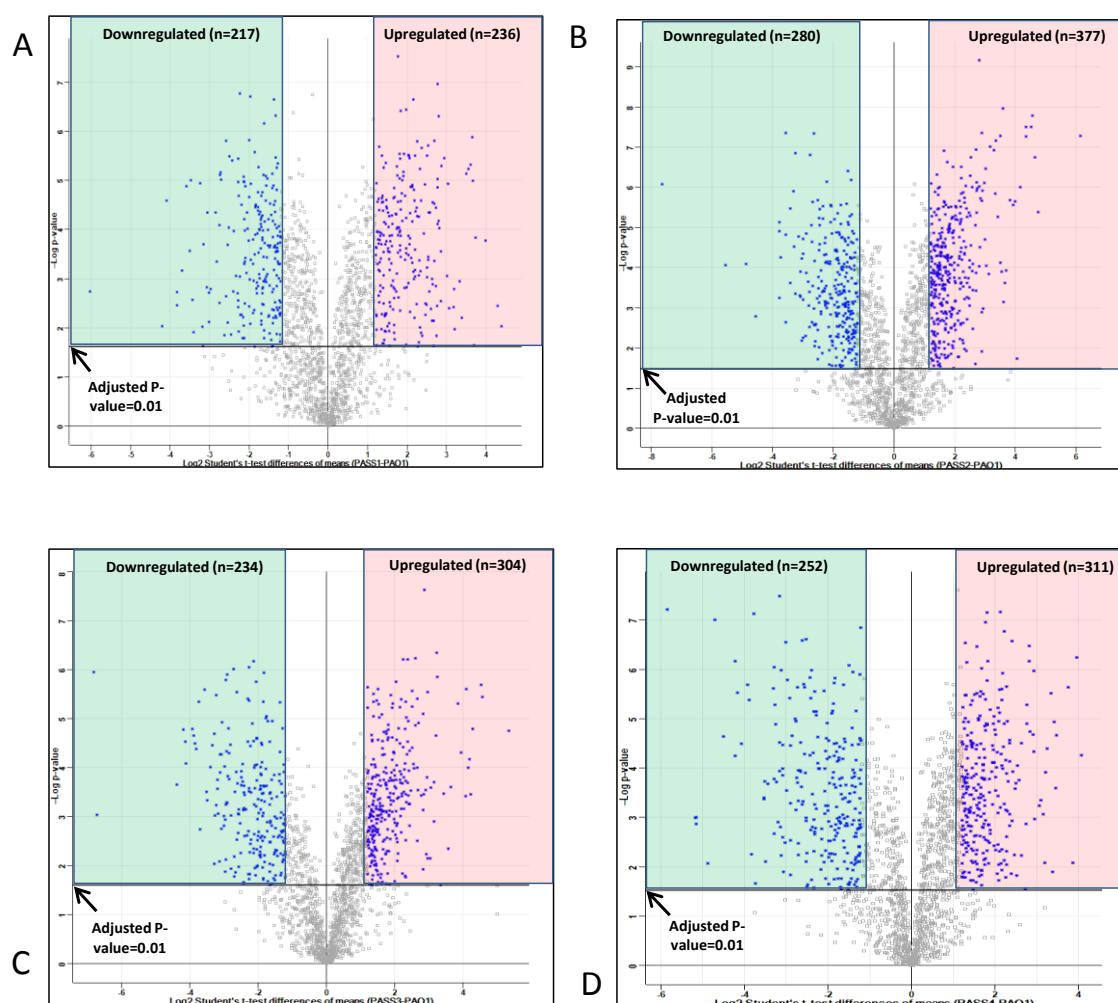
Figure-9.5: Volcano plot of the −log p-value versus the log$_2$ Student's t-test differences of mean. Each square/star represents one of the 1230 proteins. Blue starts represent differentially expressed proteins with adjusted p-value cut-off of 0.01 and log$_2$ Student's t-test fold change ±1.2 in (B) PASS1, (C) PASS2, (D) PASS3, and (D) PASS4. Blue starts in the green shaded area represent down-regulated proteins with a fold change <-1.2. Blue starts in the red shaded area represent up-regulated proteins with fold change >1.2.

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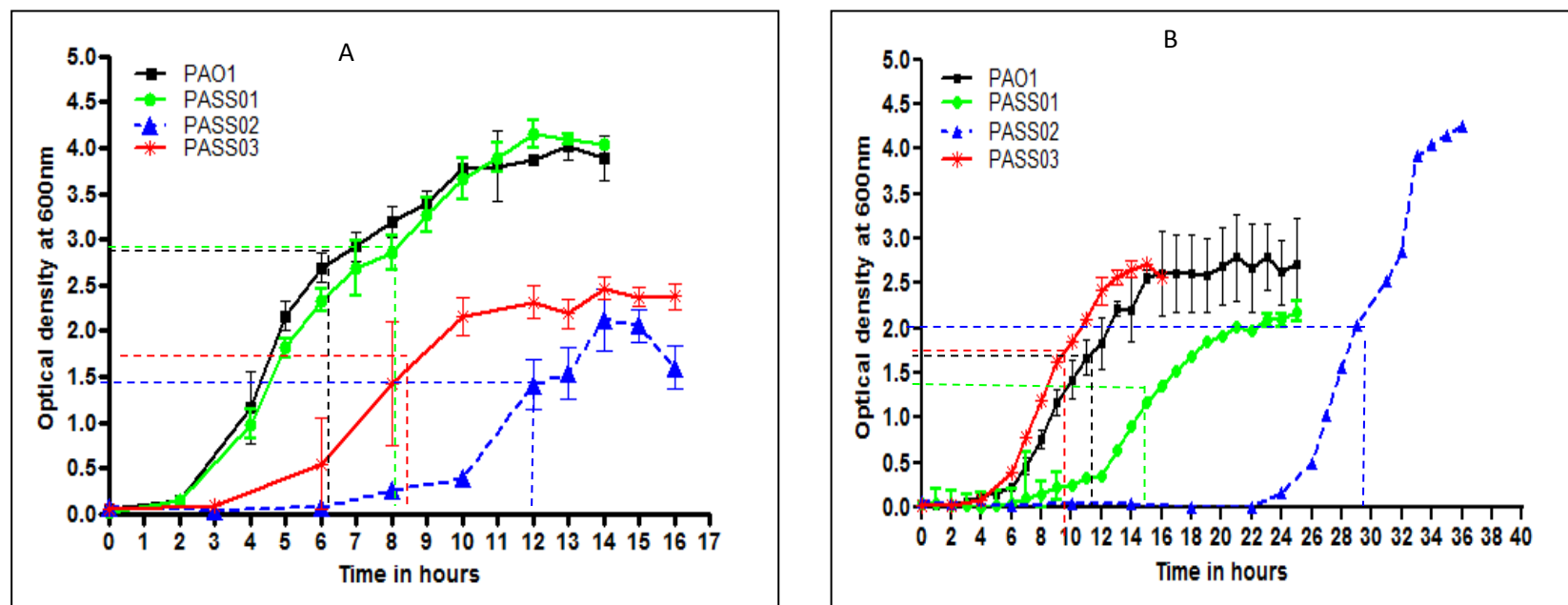
Figure-9.6: Growth curves for *P. aeruginosa* PAO1, PASS1, PASS2 and PASS3 grown in SCFM medium (A) and M9-Minimal medium (B). Dotted lines represent mid-log harvest points. (Source: Supplementary Figure S1 from Kamath *et.al* (222)).
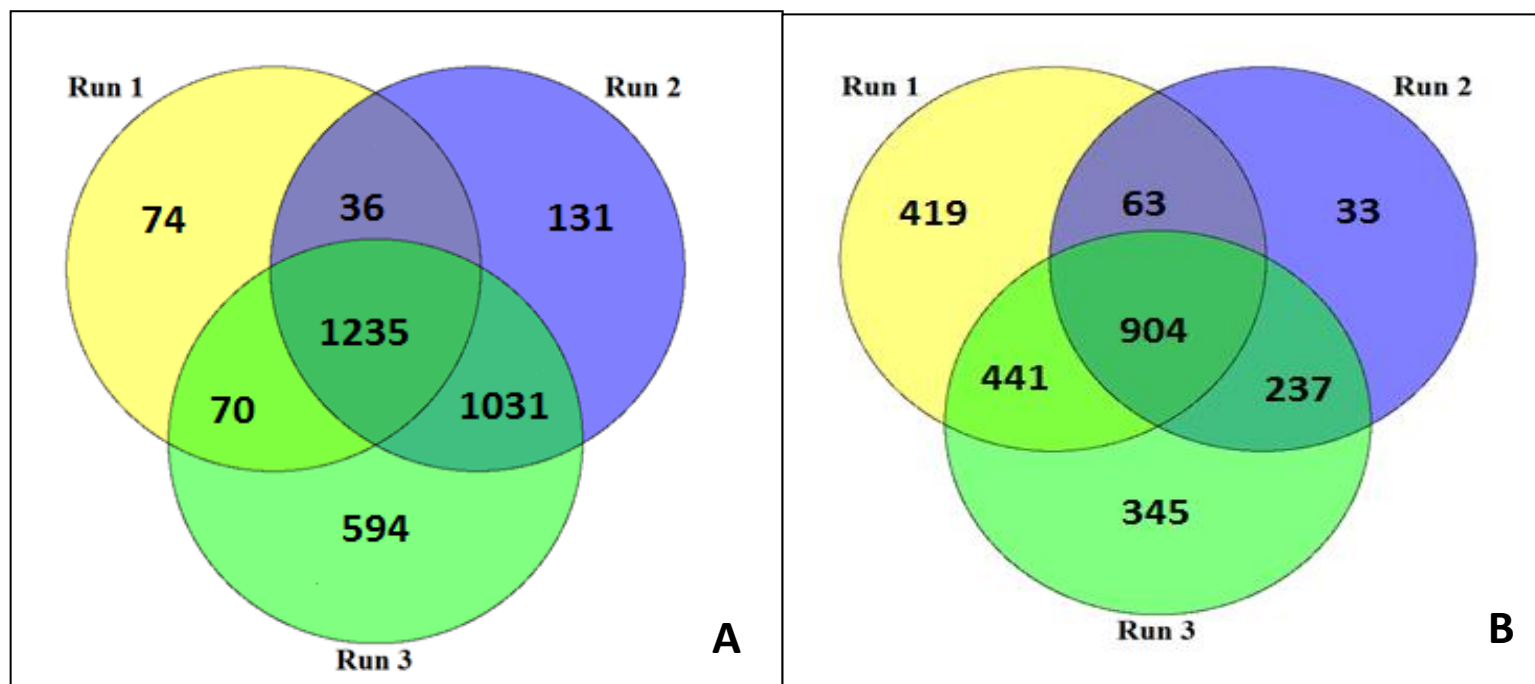
Figure-9.7: Run to run reproducibility of 2D-Nano-LC iTRAQ-MS/MS identification of proteins (*n*=3) isolated from *P. aeruginosa* grown in M9-glucose minimal medium (A) and SCFM medium (B) (Source: Supplementary Figure S2 from Kamath *et.al* (222)).

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PASS01    ----------MKAQKGFTLIELMIVVAIIGILAAIAIPQYQNYVARSEGASALATINPLK 50
PASS03    ----------MKAQKGFTLIELMIVVAIIGILAAIAIPQYQNYVARSEGASALATINPLK 50
PAO1      ----------MKAQKGFTLIELMIVVAIIGILAAIAIPQYQNYVARSEGASALATINPLK 50
PASS02    MAYTYHMEIFMKAQKGFTLIELMIVVAIIGILAAIAIPQYQDYTARTQVTRAVSEISALK 60
                    *********************************:*.**:: : *:: *..**

PASS01    TTVEESLSRGIAGSKILIGTTASTADTTYVGIDEKANKLGTVAVDIKDTGDGTVKFTFAT 110
PASS03    TTVEESLSRGIAGSKILIGTTASTADTTYVGIDEKANKLGTVAVDIKDTGDGTVKFTFAT 110
PAO1      TTVEESLSRGIAGSKIKIGTTASTATETYVGVEPDANKLGVIAVAIEDSGAGDITFTFQT 110
PASS02    TAAESAILEGKK-LVSKDNPADGEYDLGFTKSTLLAGNDGKAQITITGESSATPTIAGTL 119
          *:.*.:: .*         ..:. .        :.       *.: *   : *  .  .   .::

PASS01    GQSSPKNAG-TAITLNRTAEGVWTCTSTQEEM-----FIPKGCNKP 150
PASS03    GQSSPKNAG-TAITLNRTAEGVWTCTSTQEEM-----FIPKGCNKP 150
PAO1      GTSSPKNAT-KVITLNRTADGVWACKSTQDPM-----FTPKGCDN- 149
PASS02    GNSAGKAISGAVITIKRSAEGVWTCAISGSPANWKANYAPANCPKS 165
          * *:. *     .**::*:*.*:.***.*    :. :        : *  .* :
```

Figure-9.8: (Source: Supplementary Figure S3 from Kamath *et.al* (222)): Alignment of PilA protein from PASS1-3 stains and PAO1 performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). PilA protein had highest sequence similarity of 81% with PASS1 and PASS3 PilA protein sequence but least with PASS2 with 40% similarity.
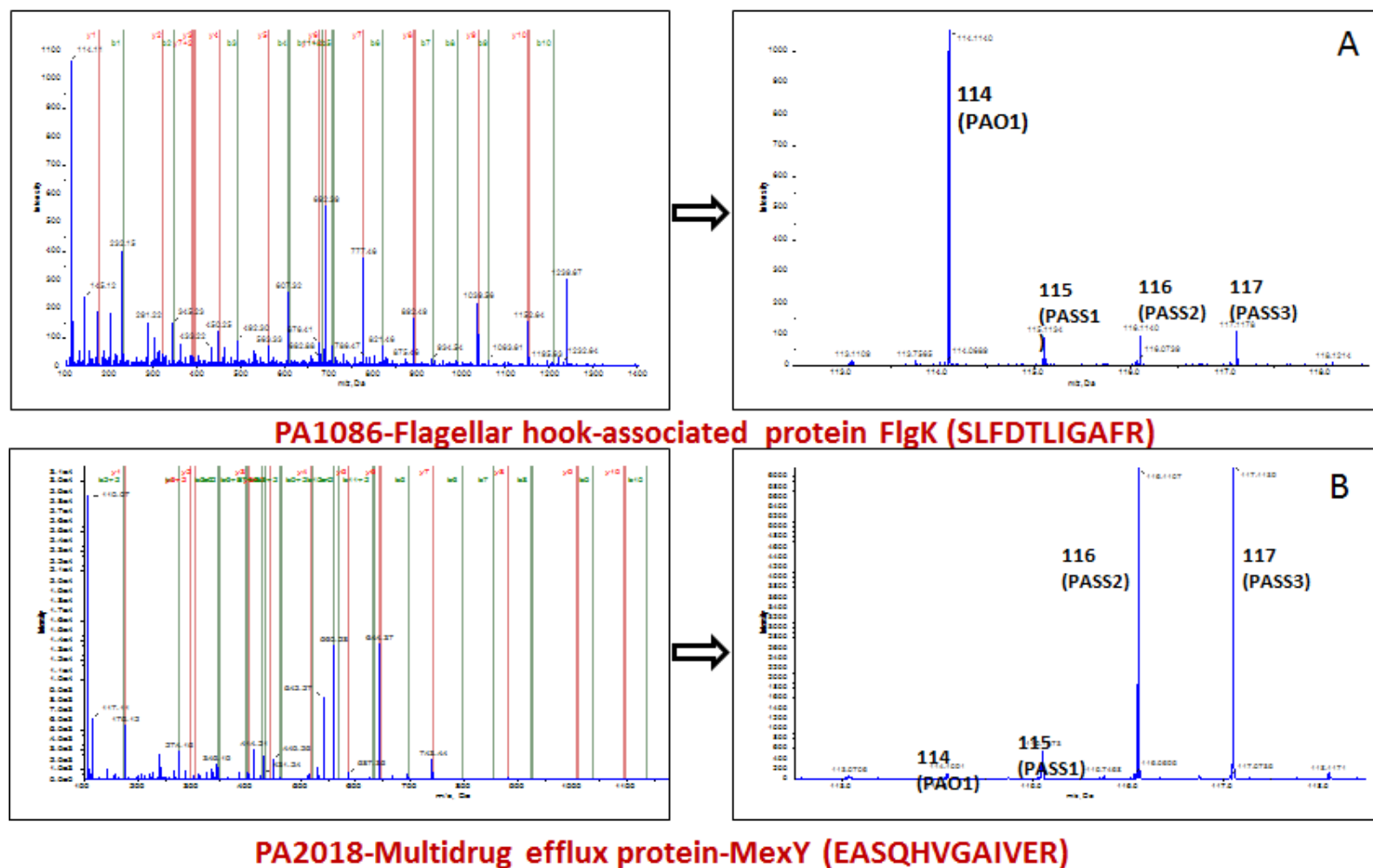
Figure-9.9: (Source: Supplementary Figure S4 from Kamath *et.al* (222)): Representative MS/MS spectra of a few differentially expressed proteins; (A) Flagellar hook-associated protein FlgK (B) Multidrug efflux protein-MexY. Inserts showing the iTRAQ reporter ion for representative peptides in control (PAO1) vs PASS strains.

Figure-9.10: Extracted ion chromatograms (Left) and respective matched MS/MS spectra of selected proteins (Right) with single-peptide quantification: Cytochrome c oxidase, *cbb₃*-type, CcoO subunit (CcoO2, PA1556, *P. aeruginosa*, PAO1), TEMDAVVAYLQVLGTSIK (+3).
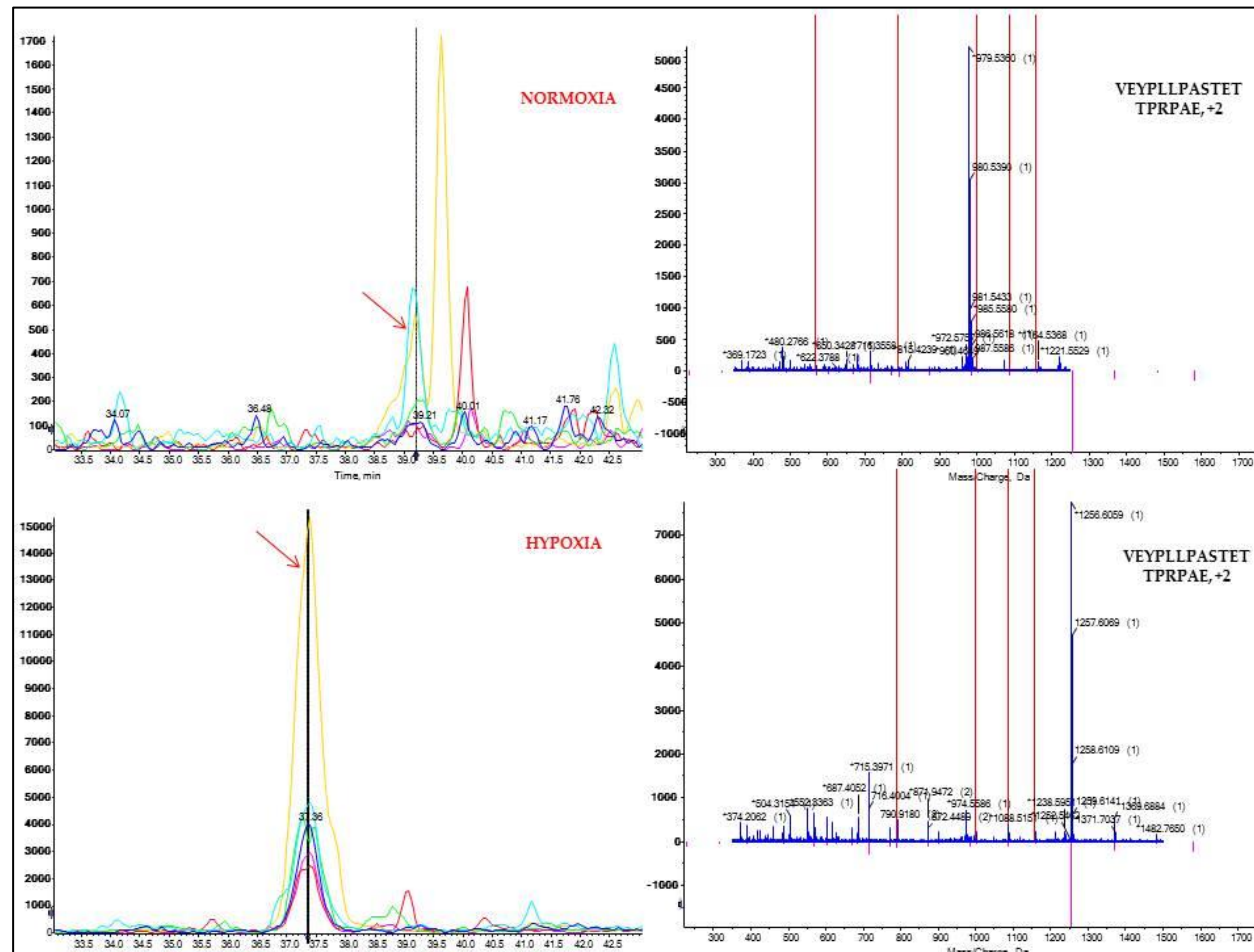
Figure-9.11: Extracted ion chromatograms (Left) and respective matched MS/MS spectra of selected proteins (Right) with single-peptide quantification: Cytochrome c551 peroxidase precursor (CcpR, PA4587, *P. aeruginosa*, PASS3), VEYPLLPASTETTPRPAE (+2)
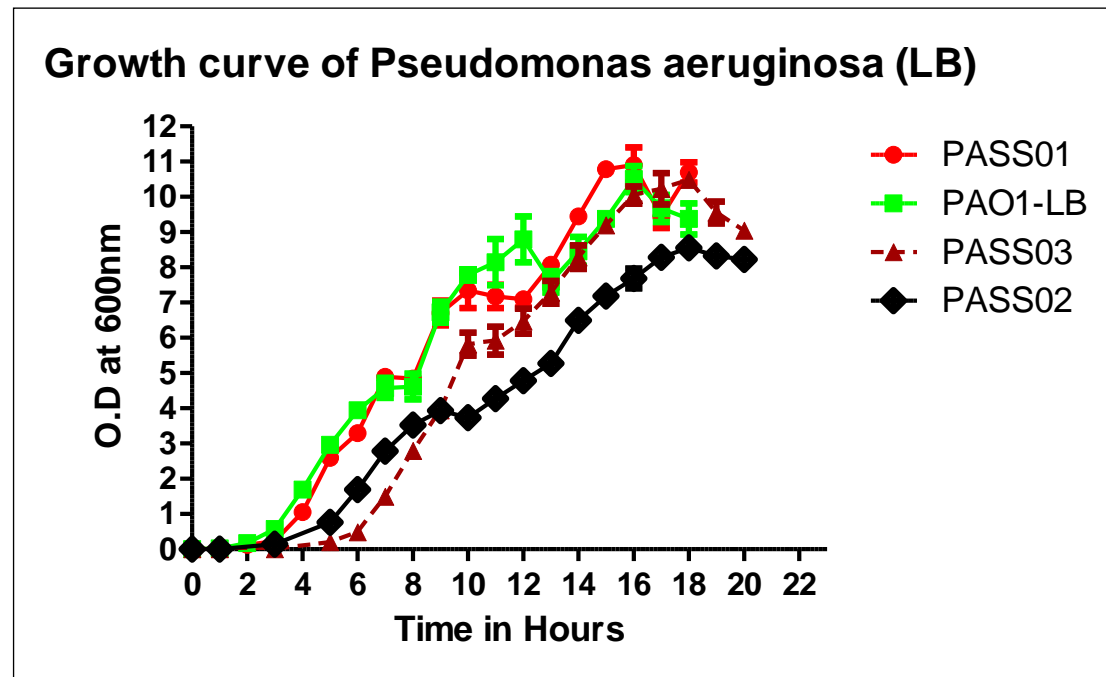
Figure-9.12: Growth curves of *P. aeruginosa* PAO1, PASS1, PASS2 and PASS3 grown in LB medium.

Table 9.1: List of pathogens of commonly isolated from lung CF patients (Adopted from Kamath *et al* (299)).

S.No	Organism	#Reported frequency (%)	Population	Reference (As per Kamath *et.al*(299))
			Bacteria	
1	*Achromobacter xylosoxidans*	8.1	Adols, adults	[34]
2	*Chryseobacterium indologenes*	5	Children	[97]
3	*Escherichia coli*	5	Children	[97]
4	*Haemophilus influenzae*	16.3	Children	[34]
5	*Klebsiella pneumoniae*	8	Adults	[97]
6	*Lysobacter enzymogenes*	4	Children	[98]
7	*Methicillin-resistant Staphylococcus aureus*	22.6	All	[34]
8	*Moraxella catarrhalis*	5	Children	[97]
9	*Nontuberculous Mycobacteria*	13	Adols., adults	[99] [34]
10	*Mycobacterium abscessus*	2	Children	[98]
11	*Mycobacterium avium*	5	Children	[97]
12	*Prevotella melaninogenica*	2	Children	[98]
13	*Pseudomonas aeruginosa*	59	Alldatabase	[99]
14	*Staphylococcus aureus*	60	Children, adols.	[34]
15	*Stenotrophomonas maltophilia*	8.1	Adols., adults	[34]
16	*Streptococcus mitis* group	8	Children	[98]
17	*Streptococcus pneumoniae*	5	Children	[97]

S.No	Organism	#Reported frequency (%)	Population	Reference (As per Kamath *et.al*(299))
		Fungi		
18	*Aspergillus fumigatus*	6-60%	All	[34]
	Aspergillus (non- *fumigatus*)	26.1	All	[100]
19	*Aspergillus spp.*	3.2-69.6	Adols, adults	[83] [101] [80] [102]
20	*Candida albicans*	78%	Children, adults	[34]
21	*Exophiala dermatitidis*	1-16%	Children, adols, adults	[34] [103] [104]
22	*Other dermatiaceous fungi (Cladospora, Curvularia and Alternaria)*	1.8-20.3	Unknown	[83] [101]
23	*Paecilomyces spp.*	3.2-8.7	Children, adols, adults	[83] [101]
24	*Penicillium spp.*	8.3-20.3	Adults	[83] [101] [86] [105]
25	*Rhizopus spp.*	0.9-1.4	Unknown	[83] [101]
26	*Scedosporium spp.*	14.7-17.4	Adults	[83] [101]
27	*Trichosporon spp.*	2.1	All	[100]
		Virus		
28	*Adenovirus*	2	Children	[34]
29	*Influenza A*	3	Children	[34]
30	*Parainfluenza virus*	6	Children	[106] [34]
31	*Respiratory syncytial virus*	2	Children	[106]
32	*Rhinovirus*	87	Children	[34]

Adols: Adolescent. * references have been provided in reference section of Kamath *et.al* (299) in chapter-2,
#Frequency: is relative frequency as reported in the respective cited article.

Table-9.2: Proteomic analyses performed on CF associated pathogens:

Organism	Method	Key findings	Reference. (As per Kamath *et.al* (299))
Bacteria	2DE, MALDI-TOF	First *P. aeruginosa* 2DE membrane proteome	[43]
Pseudomonas aeruginosa (PA)	LC-MS/MS	First *P. aeruginosa* membrane proteome using LC-MS/MS	[107]
	LC-MS/MS, iTRAQ	LC-MS/MS based analysis of clinical isolates of *PA*	[44]
	LC-MS/MS	Showed the involvement of bacterial MMPs in cleavage of CXCR1	[45]
	LC-MS/MS, iTRAQ	First proteomic study involving lung mimicking medium	[52]
	LC-MS/MS, SRM	Secretome analysis of *PA* grown in lung mimicking condition	[54]
	2DE, MALDI-TOF	Proteomic investigation of QS in *PA*	[56]
	2DE, MALDI-TOF	Proteomic study on effects of Magnesium limitation on *PA* growth	[47]
	LC-MS/MS, ICAT	Illustrated increased synthesis of PQS as clue to adaptation to CF by *PA*	[48]
	2DE, MALDI-TOF, transcriptomics	Investigation of mechanism of NO3- and NO2- respiration of *PA*	[50]
	2DE, MALDI-TOF	Proteomic analysis of adaption strategies of *PA* to anaerobic conditions	[108]

Organism	Method	Key findings	Reference. (As per Kamath *et.al* (299))
Pseudomonas aeruginosa	LC-MS/MS	Comparison of proteome between biofilm and planktonic mode of growth	[58]
	2DE, MALDI-TOF	Immunoproteomics approach to analyse the responses of human patients to secreted PA proteins	(300)
Burkholderia spp		Proteomic comparison of strain isolated from CF patients and an environmental strain	[58]
	2DE, MALDI-TOF	Proteomic comparison of clonal isolates of *B. cenocepacia*	[62]
	2DE, MALDI-TOF	Generated a 2DE proteome map of *B. pseudomallei*	[109]
	ESI-LC-MS/MS	Comprehensive map of outer membrane protein of *B. pseudomallei*	[64]
	2DE,ESI-LC-MS/MS	Identification of surface proteins of *B. pseudomallei*	[63]
	2DE, MALDI-TOF MS	Immuno reactive proteins in secreted proteins of *B. cepacia* were identified	[66]
	2DE, MALDI-TOF MS	Immuno reactive proteins in secreted proteins of *B. pseudomallei* were identified	[110]
	2DE, MALDI-TOF MS	Investigated changing secretome along with growth phase	[67]

Organism	Method	Key findings	Reference. (As per Kamath *et.al* (299))
	2DE, MALDI-TOF MS, LC-MS/MS	Characterization of survival strategies of *B. cenocepacia* in -murine model	[111]
	ESI-LC-MS/MS, iTRAQ, Gene expression microarray	Transcriptome and proteome comparison of mucoid and non-mucoid strains of *B. cenocepacia*	[70]
	ESI-LC-MS/MS	Role of type 2 secretion system in secretion of proteins in *B. pseudomallei*	[68]
	Yeast two-hybrid assay	Identified 3 novel virulence factor candidates in *B. mallei*	[112]
	ESI-LC-MS/MS	Elucidation of drug efflux pumps responsible for multidrug resistance in *B. thailandensis*	[72]
	LC-MS/MS, RT-PCR	Proteome profile of Chitosan resistance in *B. cenocepacia*	[73]
	2DE, MALDI-TOF MS	Components of RpoS regulon in *B. pseudomallei* were characterised	[113]
	2DE, MALDI-Q-TOF MS	Components of RpoE regulon in *B. pseudomallei* were characterised	[114]
	2DE-DIGE, MALDI-TOF/TOF	Investigation of proteome changes in relation to drug resistance of *B. cenocepacia*	[53]

Organism	Method	Key findings	Reference. (As per Kamath *et.al* (299))
	LC-MS/MS, qPCR	Transcriptome and proteome analysis of mico-oxic adaption of *B cenocepacia*	[75]
	2DE, MALDI-TOF-MS, RT-PCR	Role of morphotype switch was linked to survival strategies of *B. pseudomallei*	[115]
	2DE, MALDI-TOF/TOF	Investigation of small colony variance in relation to invasion, adherence of *B. pseudomallei*	[116]
	2DE, MALDI-TOF/TOF	Components of Cepl regulon governing quorum sensing were investigated	[60]
	iTRAQ, ESI-LC-MS/MS, transcriptomics	Deciphered role of CepR regulon in governing biofilm formation in *B. cenocepacia*	[77]
	2DE, MALDI-TOF/TOF	Immunoproteomic analysis of proteins expressed by four clinical strains of *B. multivorans* and *B. cenocepacia.*	(301)
	2DE, MALDI-TOF/TOF	Intra-, surface-associated and extracellular proteome of *B. cenocepacia* strain H111	(302)
Fungi	2DE, LC-ESI-TOF-MS, MALDI-TOF-MS, RT-PCR	First proteomic study on effects of hypoxia on *A. fumigatus*	[117]
	2DE, LC-MS/MS, QRT-PCR	Proteomic study revealed, hypoxia increases TCA cycle, glycolysis	[91]

Organism	Method	Key findings	Reference. (As per Kamath *et.al* (299))
Aspergillus fumigatus	2DE, MALDI-TOF/TOF, RT-PCR	Proteomic study revealed TCA-clycle enzymes down-regulation as a response to hypoxia	[90]
	2DE, MALDI-TOF/TOF	Proteomic studies Identified novel oxidative stress response protein AfYap1	[118]
	2DE, MALDI-TOF/TOF	First proteomic study in relation to oxidative stress in *A. nidulans*	[119]
	2DE, LC-ESI-MS/MS, iTRAQ	Investigated effects of antifungal drug Caspofungin on *A. fumigatus.*	[93]
	2DE, MALDI-TOF/TOF, RT-PCR, Microarrays	Proteomic study on effect of Amphotericin B on *A. fumigatus*	[94]
	2DE, MALDI-TOF/TOF	Proteomic analysis of *A. fumigatus* adaptation to anaerobic conditions	[117]
	2DE, MALDI-TOF/TOF	Proteomic analysis of response to hypoxia by *A. fumigatus*	[91]

PA: *Pseudomonas aeruginosa.*

B. pseudomallei, B. thailandensis and B. mallei are not part of BCC complex.

Table-9.3: MLST allelic profiles and strain types of *P. aeruginosa* isolates obtained from the CF sputum in this study (Source: Supplemental table S1 from Peneysan *et al* (138)).

	acs	aro	gua	mut	nuo	pps	trp	ST
PASS1	17	5	11	72	3	4	3	new
PASS2	16	5	30	72	4	13		new
PASS3	17	5	11	72	3	4	3	new
PASS4	11	84	11	3	4	4	7	649

Table-9.4: Summary of features of *P. aeruginosa* strains utilized in this study (Source: Supplemental table S1 from Kamath et.al(222))

Strains	Age	Sex	Source	Predicted ORFs	Pigmentation on SCFM-agarose plate	Patient's CF genotype	Antibiotics used for patient treatment
PASS1	40	Female	Sputum	5792	Green	ΔF508/ΔD1507	Ciprofloxacin, Tobramycin and Polymyxin
PASS2	27	Male	Sputum	5795	No colour	ΔF508/ΔF508	Polymyxin, Azithromycin, Itraconazole
PASS3	23	Male	Sputum	5847	Green	ΔF508/ΔF508	Clarithromycin, Moxifloxacin
PAO1	-	-	Wound	5571	Green	-	

Table-9.5: Volume of 100mM amino acid stock solutions added into SCFM medium (Source: Supplemental table S2 from Kamath *et.al*(222))

S.No	Component	Volume in ml
1	100mM L-Aspartic acid	8.27
2	100mM L-Threonine	10.72
3	100mM L-Serine	14.46
4	100mM L-Glutamic acid hydrochloride	15.49
5	100mM L-Proline	16.61
6	100mM Glycine	12.03
7	100mM L-Alanine	17.8
8	100mM L-Cystine	1.6
9	100mM L-Valine	11.17
10	100mM L-Methionine	6.33
11	100mM L-Isoleucine	11.2
12	100mM L-Leucine	16.09
13	100mM L-Tyrosine	8.02
14	100mM L-Phenylalanine	5.3
15	L-Ornithine monohydrochloride	6.76
16	100mM L-Lysine	21.28
17	100mM L-Histidine monohydrochloride monohydrate	5.19
18	100mM L-Tryptophan	0.13
19	100mM L-Arginine	3.06

Table-9.6: Pairwise comparison of percentage of shared genes between the PASS strains and PAO1, AES-1R strains. Colour coding indicates percentage similarity between the strains.

	PAO1	PASS1	PASS2	PASS3	PASS4	AES-1R
PASS1	89.12	100	86.67	92.23	90.5	74.05
PASS2	88.18	86.67	100	86.4	89.94	73.3
PASS3	88.8	92.23	86.4	100	89.41	73.02
PASS4	92.28	90.5	89.94	89.41	100	78.01
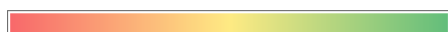
73%   100%

Table 9.7: Supplementary data files (DVD contents): The following data files are enclosed in the attached DVD.

File Name	File Type	Description	Source
Supplementary data file 3A	Microsoft Word	Proteins identified in strains PASS1-4 and PAO1 grown in LB medium via proteomics ($p < 0.01$).	Chapter-3 (Penesyan *et al* (138)).
Supplementary data file 4A	Microsoft excel	List of proteins differentially expressed in PASS1-4 in comparison to PAO1 determined by SWATH-MS. (Log_2 fold change ±1.2 and p-value<0.01)	Chapter-4
Supplementary data file 5A	Microsoft excel	List of all the *P. aeruginosa* proteins identified and their abundances.	Chapter-5 (Kamath *et.al* (222))
Supplementary data file 5B	Microsoft excel	Comparison of differentially expressed membrane protein abundance (*n*-fold change).	Chapter-5 (Kamath *et.al* (222))
Supplementary data file 6A	Microsoft excel	All identified and differentially expressed (Log_2 fold change ±1 and p-value<0.05) *P. aeruginosa*, PASS2-3 and PAO1 proteins on exposure to hypoxic stress compared to normoxic baseline.	Chapter-6

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